Microglia in the developing nervous system

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THE 'THIRD' ELEMENT

Microglia In The Developing Nervous System

DOCTORAL RESEARCH THESIS

by

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Summary

The central nervous system (CNS) contains several populations of mononuclear phagocytes whose functional roles as 'immune sentinels' involve host defence and tissue repair. Microglia are the principal mononuclear phagocytes residing within the CNS, and represent a heterogeneous population of cells in the adult and neonate. The origin of these cells was a subject of contention for most of the 20th century, a topic which is presented within this work. The consensus opinion regards the origin of microglia as derived from circulating haematopoietic progenitors that initially colonise the developing nervous system around the time of birth in rodents. There is additional indication that microglial progenitors may arise prior to vascularisation of the CNS, principally from the surrounding mesenchymal tissue. Subsequently, these progenitors disperse ubiquitously throughout the CNS, undergo progressive differentiation and establish distinct non-overlapping territories during postnatal development.

The aims of this investigation were to determine (i) the phasing and patterns of microglial invasion, (ii) mechanisms by which microglial progenitors colonise the brain and spinal cord during embryogenesis and early neonatal life, (iii) signals which may direct the migration of these precursor cells and affect their distribution primarily in man, and to a lesser extent in murine development. The following protocols were adapted for use in this study: single and dual-label immuno- and lectin-histochemistry on fresh-frozen, paraffin-embedded and vibratomed sections, assessed using light, fluorescence and confocal microscopy; Stamper-Woodruff adhesion assay with THP-1 human monocytic cell line; *in situ* hybridisation and TUNEL assay of apoptosis on cryostat sections; human foetal astrocyte and microglial co-cultures; time-lapse video microscopy and computerised image analysis; migration assays using a modified Dunn chemotaxis chamber, and electrophysiological characterisation of mixed glial cultures. Microglial progenitors were identified *in situ* in the human foetal brain and spinal cord and in culture using a panel of markers directed at monocytes and macrophages: CD11b/CD18 (Mac-1, C3bi receptor), CD45 (LCA), CD64 (FcγRI), CD68 (PG-M1/KP-1), HAM-56, and lectins RCA-1, TL and GSB-4. Human and murine foetal CNS tissues were screened for the expression of (i) adhesion molecules: ICAM-1, ICAM-2, VCAM-1, PECAM, E- and P-selectins; (ii) chemokines: MIP-1α, MIP-1β, MCP-1, MCP-3, IL-8, RANTES, IP-10, SDF-1, fractalkine, and (iii) chemokine receptors: CCR2, CCR5, CXCR1, CXCR4.

Microglial colonisation of the developing nervous system, which commences around 12 gestational weeks (12GW) in man and from embryonic day 15 (E15) onwards in mice, proceeds in a well co-ordinated manner. Microglial differentiation corresponds with highly vascularised regions of the CNS, and these cells co-localise temporally and spatially with differentiating GFAP-positive astrocytes. The interchangeable, morphological continuum and motility of microglia have been demonstrated clearly by time-lapse analysis in co-culture with astrocytes. Developmental expression of ICAM-2 on CNS endothelium, together with the spatio-temporal expression of chemokines such as MCP-1, MIP-1α, IL-8, RANTES and fractalkine, have been identified as potential signals directing microglial colonisation within the human foetal CNS. Questions related to the origin of microglia, potential signals driving their distribution, differentiation and ramification within the CNS, and their possible roles during development, are comprehensively discussed.

IN MEMORY OF THE DISTINGUISHED NEUROHISTOLOGISTS & EMBRYOLOGISTS, WHOSE PIONEERING EFFORTS HAVE CONTRIBUTED TOWARDS OUR PRESENT WEALTH OF KNOWLEDGE

DEDICATED

to my parents and to my wife and sons

to my mentor, ***Professor David Male***
for allowing me to pursue my independent research
for his invaluable guidance, patience, enthusiasm and advice
for his example of excellence in academic pursuits

to ***Professor Peter Lantos***
for his unfailing professional and personal support
and for enabling the work to be undertaken in his Department

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Institute of Psychiatry, London, within the UK and worldwide,
for their ongoing collaborations, encouragement and support

"*According to classical ideas, nerve tissue is formed of two kinds of cells which differ in form and function-namely, nerve cells and neuroglia cells. It has been necessary to admit, in addition, the existence of a third group of cells which I have called microglia.*"

"*The microglia, or third element, although it is not a part of the fundamental architecture of the central nervous system, is always present in nerve tissue; indeed it is intimately associated with its elements for the auxiliary, but nevertheless necessary, task of taking up, transforming and removing the products of normal metabolism and abnormal disintegration of neurons.*"

"*Which are the elements of the pia-mater that, on entering the nerve tissue, give rise to microglia I do not know with certainty. Present histological methods do not detect the initial stages of transformation of microglioblasts. Nevertheless in the pia and adventitial connective tissue nuclei resembling lymphocytes are present in large numbers in those places where the microglia is formed, and I am inclined to believe that those nuclei are the mother elements of microglia. The lymphocyte-like bodies corresponding to the polyblasts of connective tissue, immigrate into the nerve tissue, and this might explain why microglial cells are able to develop migratory activities and amoeboid and pseudopodial movements, and may be converted into macrophages.*"

"*These are, in brief, my observations on the normal characters of microglia. They have been amply confirmed, although some authors have been slow to accept my ideas. It is always difficult to replace deeply rooted ideas, and some fundamental conceptions such as the mesodermic origin of microglia, are still under discussion. Embryological observations, however, demonstrate its mesenchymatous nature and they are supported by the physiological observation that it behaves as a mesodermal element . . .*"

"*These ideas have been developed in my papers from 1919 to 1921, and though they have been discussed in many subsequent papers by other workers, almost nothing new has been added to our knowledge of this subject. Today these ideas are widely accepted.*"

Pio Del Rio-Hortega [*from a lecture delivered at Oxford University on Nov. 2, 1938*]

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Foreword

For the past two decades, microglia have been widely regarded to originate from circulating blood-derived bone marrow elements which appear in the nervous system prior to and shortly after birth, migrate to all constituent parts of the CNS, and remain in these localities in an 'inactive' or 'resting' state until the advent of some disease. Furthermore, it is recognised that one of the first noticeable signs in any form of pathology affecting the nervous system is a tremendous increase in the activity of microglia, which proliferate and migrate to the site of damage and phagocytose cellular and other débris. Once the affected area is cleared of potentially harmful matter, astrocytes have the opportunity to lay down a scar in place of the destroyed tissue. These descriptions have held true to a great extent until now. Nevertheless, evidence that has recently emerged from several independent sources, including the present work, indicates possible differences in the lineage of microglia which arise during the foetal period (derived from foetal macrophages or of mesenchymal/myeloid origin) from those that may populate the nervous system in the neonate and in the adult (derived from circulating bone marrow progenitors). The chapters that follow will focus primarily on studies on the colonisation of the developing human and murine nervous systems by microglia *in situ* (Chapter II & III), characterisation of human foetal microglia and astrocytes *in vitro*, and the interaction between these two cell types in tissue culture (Chapter IV). The thesis will close with a discussion of the findings with respect to current hypotheses on the origin of microglia, and their potential roles within the developing nervous system (Chapter V).

Several main concepts will be discussed within the context of this work:

1. Microglia initially arise from progenitors located in the mesenchymal tissue surrounding the embryonic CNS, and concurrently from circulating mononuclear progenitors (foetal macrophages) that access the CNS via the bloodstream and vascular endothelium, at early stages of foetal life.

2. The precise route of invasion into the CNS (i.e. vascular or meningeal/connective tissue associated) is dependent upon (i) the specific period in embryonic/foetal or postnatal development; (ii) the specific location within the central nervous system, and (iii) the particular species under investigation.

3. Dissemination of microglia throughout the CNS is a progressive, co-ordinated event that appears to be temporally and spatially regulated by a variety of signals derived from the CNS tissue itself, but not specifically restricted to neuronal apoptosis (naturally occurring programmed cell death).

4. During development, the transformation of 'naive' microglial progenitors, into the 'mature' ramified forms is most probably under the direction of diffusible, as well as contact-mediated factors derived from within the CNS, including the extracellular matrix components with which these cells come into contact. However, *in vitro* experiments indicate that the various morphological states of microglia are interchangeable, and the same cell may undergo rapid and reversible transformation from one state to the other, with transitional (intermediate) morphologies frequently encountered.

5. Experiments which manipulate the influx, differentiation and distribution of foetal mononuclear phagocytes into various embryonic organs (including the skin, liver, lymph nodes, and lungs), with particular focus on the CNS (for example through knockout and transgenic technology using myeloid transcription factors) will help to further clarify the origins, biological functions and potential involvements of these different subpopulations in the histogenesis and modelling of tissues during development.

6. The findings of the present work have direct bearing for the clinical therapy of a number of hereditary paediatric neurological disorders (hereditary lipidoses, Gaucher's disease, Krabbe's disease) which are associated with a deficiency in certain key enzymes within the CNS, as well as defective mononuclear phagocytes. Transplantation using bone marrow and cord blood (rich in haematopoietic progenitors) has proven partially successful as a form of clinical treatment. The use of microglial progenitors as a tool for gene therapy to target the CNS in such disorders is now conceivable. This study has shown that an effective window for such therapy exists during the second trimester of intra-uterine human life, commencing with the infiltration of progenitors around the twelfth week of gestation.

Chapter I INTRODUCTION & OVERVIEW

A brief synopsis of the history behind the discovery and origin of microglia

Prior to the twentieth century, the nervous system was considered to be composed of two types of cell, which differed in form and function. namely nerve cells and neuroglia (interstitial cells of the nervous system). Following the discovery of neuroglia by Virchow in 1846, numerous attempts were made to demonstrate these cells *in situ*. However, belief had been prevalent for some time that mesodermic elements also penetrated the nervous tissue both during embryonic development and under pathological conditions, and could subsequently transform to a population of neuroglia (see Rezaie and Male, 2002b for references). Scholars of the late 19th century generally adhered to three principal theories regarding the origin of 'neuroglia' as being derived: (i) solely from ectoderm, or otherwise from the primitive medullary canal, (ii) in equal part from mesoderm and ectoderm, or (iii) from the mesoderm. The idea that neuroglia in man could be derived from mesodermal tissue was further emphasised by Eichhorst in 1875. He noted that 'neuroglia' were absent from the white matter of the spinal cord until the fourth month of foetal human life, when extravasating leukocytes began to migrate and ramify, and became immobile upon reaching their final destinations. The terms 'mesoglia' and later 'third element of the nervous system' were first introduced by W. Ford Robertson (1900) and Santiago Ramon y Cajal (1913a,b) respectively, to define such phagocytic mesoderm-derived elements within the nervous system, taking into account their separate origins from neurons and neuroglia (a term predominantly designating astrocytes). This was later amended by del Rio-Hortega between 1919 and 1921, to 'microglia' in order to further discriminate between true mesodermal elements and oligodendrocytes, previously regarded as a component of 'mesoglia'. This particular contention sparked much controversy among del Rio-Hortega's peers and resulted in an escalation of fruitful research throughout Europe that eventually declined up to the outbreak of the Second World War. The post-war years were a period during which the very existence and nature of microglia were cast in doubt until, in the 1960s, a new cohort of investigators realised the potential that is now commonly ascribed to microglia as 'intrinsic immune effector cells of the CNS'.

The discoveries of Pio del Rio-Hortega

Although del Rio-Hortega's original descriptions of the morphology and function of microglia have been confirmed time and again, the origin of these cells has proved more controversial. Based on his studies in laboratory animals, del Rio-Hortega stated that '*according to all indications, the microglia is formed through the migration of embryonic corpuscles from the*

pia into the nerve centres' and additionally *'from other related elements, chiefly the blood mononuclears'*. He also defined the time-frame for colonisation of the mammalian nervous system, to occur late during embryonic development and after birth (coinciding with vascular and meningeal organisation). del Rio-Hortega noted that there was unequal distribution in the quantity of microglia at different stages of development, with cells in some areas forming large masses (in the deeper portions of the hemispheres, along the path of vessels and spreading under the ependyma), but entirely absent in others. He defined the major sources of microglia within the interhemispheric commissure, related to the meningeal fold entering the brain via the fissure of Bichat and ascending towards the lateral ventricles, as originating from [i] the superior tela choroidea and [ii] pia covering the cerebral peduncles and cerebellum. He further defined pial adventitiae of large and medium-sized blood vessels as additional foci. "*Within a few days all these regions supply the brain with very numerous microglial elements that migrate through the nervous interstices and spread in diverse directions. As the migration radiates towards the convolutions, the microgliocytes soon invade areas far removed from their source: the cells which originated in the superior tela choroidea enter the corpus callosum above and the cerebral trigonum and optic thalami below spreading throughout the ammonic cortex. They migrate towards the walls of the ventricles and later penetrate the white matter of the hemispheres finally reaching the cerebral cortex. The microglia entering through the region of the peduncles, where it appears at first as a dense marginal layer, rapidly ascends to the region of the internal capsule and is distributed throughout the hemispheres. In its migration it follows, more or less closely, the direction of the vessels.*" (del Rio-Hortega, 1932). These significant observations have been overlooked by many authors who have referred to del Rio-Hortega's work in the past.

Del Rio-Hortega also described the various morphological forms of microglia, and introduced the concept that the degree of motility of these cells was related to alterations in their shape. He described early rounded forms of microglia which occurred in loosely-packed structural zones (for example in the white matter of cerebral hemispheres and in the 'interammonic' region between the hippocampi, where they either formed dense masses, were scattered or arranged in rows along blood vessels. Numerous processes of various length and calibre projected from their cell body, and these became more elongated and finely branched as they encountered the dense framework of the cortical plate (future neocortical grey matter) with more narrow interstices. Upon reaching their final destinations microglia adopted even finer and longer processes, later to be divided into secondary and tertiary branches with retractile spines which fit into the interstitial space. On passing along closely-packed fibers such as the corpus callosum, in perivascular or perineuronal spaces, and in other neuronal tract regions (such as the corpus callosum, fimbria, cerebral peduncles and ascending nerve fibers),

microglia often appeared as elongated, bipolar and lamellar cells. Their colonisation and distribution was almost complete by a few days after birth, although 'microgliocytes' (precursor cells) could still be found in larger numbers in the white matter, where amoeboid and less ramified varieties remained for a longer period. These gradually subsided as myelination neared completion. Microglia were noted to be heterogeneous in their morphology, their shapes showing variability and possessing usually three to four, but also up to six or more, primary processes. On the whole however, microglia were typically arranged according to the general cytoarchitecture within each region of the central nervous system. Those cells located within the grey matter soon assumed multipolar branched shapes of great complexity and uniform distribution. The capability of these cells to function as phagocytes and migrate to sites of injury within the CNS under pathological conditions was a phenomenon that had already been convincingly demonstrated in the 1920s and 1930s by experiments particularly carried out by Wilder Penfield and del Rio-Hortega (see Rezaie and Male, 2002b). Most pathologists were willing to accept the notion that phagocytes in the CNS originated primarily from resting microglia (for example Cone, 1928) and secondarily from cells associated with the connective tissue surrounding blood vessels (most likely the perivascular macrophages) or from circulating blood monocytes (Gozzano, 1931a,b,c; Testa, 1929).

Histogenesis, related distribution and turnover of microglia within the CNS

Various centres of microglial proliferation were denoted as 'fountains' by del Rio-Hortega (1919a, 1921a, 1921b, 1932). Such sites included the interpeduncular fossa (the point where the invaginated pia forms the tela choroidea of the third ventricle) and a region located beneath the tela of the fourth ventricle. Microglial progenitors (often termed 'microglioblasts' or 'microgliocytes') made their appearance immediately below the pia mater, and migrated from these areas into the brain tissue. Further studies mainly in rodents, led other authors to propose that microglia were also derived from perivascular tissues in addition to elements in the pia mater (von Sántha, 1932; Winkler-Junius, 1926). In 1939 Kershman, who had been studying the human foetal brain and spinal cord, noted that the earliest influx of microglia-forming cells was concomitant with the earliest vascularisation of the developing human nervous system. At early stages of embryonic development in particular, microglial progenitors were found to migrate from these 'fountains' into the CNS along branches of choroidal and pial blood vessels (Belezky, 1932; del Rio-Hortega, 1919a, 1921a, 1921b). Progenitors located in the leptomeninges and adventitial connective tissue of blood vessels were referred to as 'polyblasts' or 'histiocytes' of the connective tissue by del Rio-Hortega, and their nuclei reported to resemble that of lymphocytes.

Gozzano's extensive studies between 1929 and 1932 on the histogenesis of microglia in newborn rabbits, confirmed del Rio-Hortega's descriptions, the areas of invasion of mesenchymal cells corroborating del Rio-Hortega's fountains. von Sántha (1932) made similar observations in the brains of cat, dog and rat embryos. Significantly, he noted that microglia could be demonstrated much earlier in development than a few days before birth. Prior to mid-gestation, he observed the presence of mature microglial cells in the nerve parenchyma, usually intimately associated with blood vessels, and their occurrence corresponded to the general development of the particular site. The following year, von Sántha and Juba (1933) demonstrated the earliest appearance of microglia in the rhombencephalon and diencephalon of 15mg rat embryos to coincide with the onset of vascularisation in these areas. Juba (1933, 1934) additionally found microglia in the brain of a human foetus 23mm in length and fowl embryo 5 days old. Dunning and Furth (1935) observed 'many mature microglia cells with fully developed processes' in the brains of chicken and guinea pig embryos, close to capillaries and located below the pia mater and the ependyma. These observations (also confirmed by Bolsi, 1936) led the authors to believe that microglia could be derived from vessel-associated connective tissue and probably from circulating blood monocytes, at a considerably earlier period of time than shortly before birth.

Two controversial questions arose from the derivation of microglial progenitors from pial elements. The first addressed the exact composition of cells forming the pia mater, and the second whether pial cells could give rise to macrophages. In his study on foetal rats, Dougherty (1944) showed that microglial progenitors spread laterally from where the pia was in contact with the corpus callosum and fimbria fornix. The areas of greatest proliferation were located to the borders of the dorsal lateral wall of the lateral ventricles, intermingled with oligodendroglial progenitors and ependymal cells. Microglia dispersed from these sites along the length of fibers of the corpus callosum dorsally and around bundles of fibres ventrally, and acquired various bipolar or spindle morphologies along the direction of the fibres. In tangentially cut fibres, microglia were found intermixed with oligodendroglia and formed rings about bundles of developing neurons. Based on nuclear morphology and structural characteristics, Dougherty noted that cells within the pia mater were similar to those present in fountains, and furthermore, that amoeboid macrophages of a similar variety could also be found in the cerebrospinal fluid (CSF) and ventricles of 17 and 18 day-old foetal rats, but not in newborn animals. Microglia within the CNS were further found to share morphological characteristics with tissue macrophages found in other parts of the body. Macrophages laden with lipid were located within the pia mater and around pial vessels in foetal animals, but were for the most part located to deeper structures within the brain of newborn rodents. Pia-forming cells had been shown to take up vital dyes, bacteria, carbon particles as well as lipids that were

found normally in newborn rats and other species. In the 17 and 18-day old foetal rats, the pial zone was filled with macrophages. However, perivascular accumulations of cells or large numbers of cells within blood vessels were not common occurrences, and could not substantiate the view that microglial progenitors present in the pia and tela choroidea were blood-derived (Dougherty, 1944; Gozzano 1930). During the course of these early studies, it was also found that pial cells which entered the brain of newborn rats were not predominantly phagocytic. Apparently, many became phagocytes following invasion of the nervous tissue.

del Rio-Hortega was well aware that arguments sparked by the controversy surrounding the origin of microglia were in part due to the difficulties in identifying microglia, and that progress in this area was limited to the techniques available at the time: "*The elements present in the pia, its folds and the vascular adventitial layers that, after entering the nervous system are transformed into microglia are at present unknown*" and furthermore "*the main difficulty in the study of this important detail is due to the imperfections of the* (silver impregnation) *technique since only two fundamentally opposite methods are available, namely, the nuclear technique and the protoplasmic staining method . . . none of the present-day techniques stains simultaneously and successively the protoplasm and the nuclei of the superficial elements.*"

This controversy was still rife nigh on half a century after the discovery of microglia, even with the introduction of electron microscopy to such studies. Microglial progenitors were once again located to developing vessels and the sub-pial border by Vaughn and Peters (1968) and Vaughn (1969). These authors however, rejected a mesodermal origin for microglia. Fujita (1965) argued that cells inside the embryonic pia mater differentiated from sub-pial glioblasts and did not migrate into the neuropil from the pia mater, but later agreed (Fujita & Kitamura, 1975) that resting microglia could be derived from mesenchyme. Reactive (that is, activated as opposed to normal resting) microglia were thought more likely to be derived from monocytes. Others considered microglia to derive intrinsically from progenitors within the CNS, from perivascular cells (Miyagawa, 1933) (now referred to as perivascular macrophages), or exclusively from blood monocytes (Kitamura, 1973; Fujita and Kitamura 1975,1976). Differences inherent between species were considered as a possibility for causing the largely contradictory findings of this period (Cammermeyer, 1970; Feigin, 1969; Hain, 1963). During the 1960s however, many authors tended to agree that 'resting' microglia represented a uniform cell population identifiable by light microscopy (Cammermeyer, 1966; Feigin, 1969; Glees, 1955; Hommes & Leblond, 1967; Ibrahim et al., 1968, 1974; Kreutzberg, 1966, 1968b; Lewis, 1968; Naoumenko & Feigin, 1963; Niessing, 1952; Scheibel & Scheibel, 1958; Sjöstrand, 1966a,b; Tsujiyama, 1963). Approximately 5-10% of all non-neuronal cell nuclei in the neuropil were estimated to represent microglia in the cerebral cortex (Brownson, 1956) the

corpus callosum (Mori & Leblond, 1969), and optic nerve (Stensaas. 1977; Vaughn & Peters, 1968).

In 1968, Roessman and Friede first addressed the question concerning the turnover and origin of microglia *in vivo*, by injecting labelled bone marrow cells into rodents. They found isolated labelled cells in the neuropil of normal and traumatised recipient animals to be indicative of a haematogenous origin for microglia. Manfred Oehmichen (1980) separately conducted a series of transfusion experiments in the 1970s. By labelling blood cells from a donor animal and transfusion into an irradiated recipient animals, he could identify labelled cells within the neuropil of a few normal adult animals, but could not determine whether the migrated mononuclear cells transformed into resting microglia. Radioactively labelled monocytes injected intravenously into healthy, brain-damaged or nerve-damaged rabbits yielded inconsistent results with very few occasional cells in the neuropil. In the late 1970s, Eng-Ang Ling (1978) also found blood-derived mononuclear cells within the brain of neonatal rats, following intravenous injection of colloidal carbon. It was further proposed (mainly through autoradiographic studies) that the majority of mononuclear cells invading the CNS could also proliferate locally (Adrian & Smothermon, 1970; Fujita & Kitamura, 1975, 1976; Kitamura et al., 1972; Konigsmark & Sidman, 1963; Smith & Adrian, 1972). Nevertheless, this view was not shared unanimously. Oehmichen (1980) estimated the labelling index for microglia to be quite poor (between 0.2-0.3%) in contrast to that for monocytes and macrophages of other organs (for example, mouse blood monocytes: 2.9%, mouse peritoneal macrophages 1.3%; mouse liver macrophages 1.5%; mouse alveolar macrophages 2%). It appeared that the frequency of division in mononuclear phagocytes from various sites in the body was up to ten times greater than for comparable elements within the CNS. A distinct increase of between 1-3% labelling index could however, be established in proliferating mononuclear phagocytes of the CNS under pathological conditions. Kitamura and colleagues (Fujita & Kitamura, 1975, 1976; Kitamura et al., 1972) in particular, maintained that microglia were derived exclusively from blood monocytes that had invaded the CNS. These could *en passage*, arbitrarily lie inside the basement membrane sheaths during migration (and could be distinguished from pericytes which were not derived from monocytes).

Still the issue regarding the origin of microglia remained unresolved. Oehmichen (1980) did not support a migration of blood cells into the undamaged neuropil. He noted that labelled migratory cells within other organs were similarly encountered only rarely under non-pathological conditions (for example in non-irritated peritoneal cavities and in the undamaged liver). The idea that transformation of migrating blood cells and intrinsic local division of CNS-resident mesodermal phagocytes were concurrent events, was one that had already been

voiced earlier in the 20th century (Achúcarro, 1904; Forster, 1908; Marchand, 1909, Merzbacher, 1910). By the 1970s, it became established that mononuclear invasion of the CNS occurred in response to inflammatory or traumatic stimuli. On the other hand. evidence for mononuclear invasion was absent in models of Wallerian (Friede & Johnstone, 1967: Skoff & Vaughn, 1971) or retrograde degeneration (Adrian & Smothermon, 1970; Berner et al., 1973; Kreutzberg, 1967, 1968a, 1968b; Sjöstrand. 1965, 1966a, 1966b; Stenwig, 1972). Instead, under these circumstances, a local proliferation of activated mononuclear phagocytes was indicated. Most of the locally proliferating cells could be demonstrated between the second and fifth days post-lesioning. Thus emerged the hypothesis that under normal conditions, the relatively slow turnover of microglia in the postnatal and adult nervous system resulted mainly from local cell division.

Since the 1970s, numerous studies have identified morphological, phenotypical and functional similarities between microglia and cells of the mononuclear phagocyte system (van Furth et al. 1972). Ultrastructural studies using transmission electron microscopy confirmed the earlier light microscopic descriptions that microglia and blood monocytes shared similarities in their morphologies (Anker, 1975; Blakemore, 1975; Das, 1976; Das & Ptaffenroth, 1976; Gonatas et al., 1964; Lampert & Carpenter, 1965; Persson, 1976; Schultz & Pease, 1959). Microglia were later shown to share phenotypic characteristics (cell surface markers) with monocytes and macrophages, but not with astrocytes and oligodendrocytes (Miyake et al., 1984; Murabe & Sano, 1983; Perry et al., 1985; Valentino & Jones, 1980; reviewed in Streit et al., 1988). Sophisticated bone marrow chimera studies in rodents demonstrated that perivascular microglia were replenished from bone marrow progenitors (deGroot et al., 1992; Flügel et al. 2001; Hickey, 1991; Hickey & Kimura, 1988; Hickey et al., 1992; Matsumoto & Fujiwara, 1987). Curiously, there were no clear indications from these studies that perivascular microglia could transform to parenchymal ramified microglia. This evidence, together with the observation that there is no perivascular infiltration of mononuclear cells in adult rodents following transection of facial motor neurons (Graeber et al., 1988), and in addition to the thymidine incorporation experiments highlighted above, has substantiated the idea that parenchymal microglia have a very low turnover in the adult CNS (Hickey et al., 1992; Lassmann et al., 1993; Lawson et al., 1992; Perry, 1994; Perry & Gordon, 1991). This contrasts with perivascular cells, which possess higher rates of turnover and occur in a more phenotypically and morphologically activated state at rest. It has been reaffirmed that the microglial population is maintained through an equal share of *in situ* division of resident cells and migration of monocytes, and estimated that it would take the lifetime of a mouse for the entire microglial population in the steady state to be replaced by immigration of monocytes alone (Kennedy & Abkowitz, 1997: Lawson et al., 1992: Yeager et al., 1992). The most

plausible explanation for all these findings would be to consider that the influx of microglial precursor cells is confined to a brief perinatal 'time-window', and any subsequent increase in the adult microglial population is predominantly a result of the proliferation of pre-existing parenchymal microglia (reviewed in Rezaie & Male, 1999). It is this perinatal window in time that will be the subject of comprehensive analysis in the present work which investigates both the human and murine CNS.

Phagocytes within the CNS and their relationship with microglia

There are at least four compartments within the central nervous system in which phagocytic cells can be found. Apart from microglia within the parenchyma of nervous tissue, there are also (i) the population of perivascular cells already mentioned, that are located between the glial and endothelial basement membranes, (ii) subarachnoidal cells and those detected within the cerebrospinal fluid (CSF), and (iii) epiplexus cells of the choroid plexus. Only the intrinsic microglia have been discussed so far. Two questions arise that require further consideration of mononuclear phagocytes within other compartments. The first relates to the extent that other cell types contribute towards microglial histogenesis within the CNS during development. The second addresses the origin of phagocytes in these other compartments: Do all mononuclear phagocytes associated with the CNS originate from circulating progenitors derived from the blood, or is there an intrinsic mesenchymatous/ adventitial origin for some populations (for example, subarachnoid 'free' cells and parenchymal microglia) ?

microglia derived from perivascular cells (?)

A clear distinction between pericytes located around capillaries, perivascular phagocytic cells (probably macrophages) and perivascular parenchymal microglia is very rarely made in the literature. This has gained considerable attention recently (Thomas, 1999), particularly with respect to repopulation of resident microglia from blood-derived progenitors. The relationship between these cell types will have a strong influence on current concepts of microglial origin, and a clarification between perivascular microglia and perivascular macrophages is necessary to avoid confusion (Graeber et al., 1989, 1990, 1992; Perry & Gordon, 1997). This is particularly important when considering the earlier views proposed by some authors that microglia could be derived from perivascular cells located on the external surface of vascular basement membranes belonging to small arteries, capillaries, venules and small veins (Baldwin et al., 1969; Cammermeyer, 1965-1970; Dunning & Stevenson, 1934; Field, 1955; Jones, 1970; Juba, 1934; Kershman, 1939; Miyagawa, 1934; Mori and Leblond 1969; Schaltenbrand & Bailey, 1928; von Sántha, 1932; Vaughn, 1965). Whether populations of microglia can indeed originate from perivascular macrophages is as an issue that is yet unresolved.

***microglia derived from subarachnoid cells* (?)**

The subarachnoid space is an extension of the intraparenchymal perivascular space. The pial surface layer of the CNS has fenestrations or openings that allow continuity between the subarachnoid and endoneural spaces. The leptomeninges (pia-arachnoid and invading vessels) have been considered by the majority of investigators to derive from mesoderm. and we have already noted del Rio-Hortega's view that microglial progenitors within the brain were derived from perivascular and pial connective tissue (mesenchyme) during the embryonic period, which following invasion of the cerebral parenchyma, transformed to microglia. In 1972, Morse and Low noted that mesenchymal cells possessing the morphological characteristics of macrophages, could frequently be observed below the pial cell layer (between the pia and glial membrane). The majority of these mesenchymal cells (both on the surface of the brain and along cerebral vessels) could undergo transformation from a quiescent to an active phase, contained lipid vacuoles and resembled microglia from a morphological sense. Migration of these cells from the edges of vessels and the leptomeninges into the neuropil along with their morphological transformation could explain these findings (Belezky, 1932; Gozzano, 1931a-c; Kershman, 1939). In a similar manner, Herrlinger and colleagues (1977) noted peroxidase-positive derivatives of monocytes located at juxtavascular ('satellite') positions within the neuropil and in the subarachnoid space.

***microglia derived from ventricular macrophages and epiplexus cells of the choroid* (?)**

Macrophage-like cells were originally noted on the surface epithelium of the choroid plexus by Goldmann in 1913 and Kolmer in 1921, who believed these elements to derive from the blood. Kolmer described the motile and phagocytic ability of these 'wandering cells'. Biondi (1934) later described them in man. The term 'Kolmer cell' was adopted for use until the suggestion by Kappers (1953, 1958) of 'epiplexus cell', to reflect the observation that these cells remained in direct contact with plexus epithelium over long periods. It was assumed that epiplexus cells originated from connective tissue of the tela choroidea and proliferated locally prior to migration into the ventricular cavity (Biondi, 1934; Vialli, 1930). Other authors described similar cells on the surface of ependymal walls, referred to as 'supraependymal cells' (Allen, 1975; Coates, 1972, 1973a, 1973b, 1975; Hosoya & Fujita, 1973; Peters, 1974; Walsh et al., 1978). It was suggested that blood monocytes reached the ventricle by migrating through choroidal vessels and epithelium of the choroid plexus.

From an early period, it was noted that large numbers of microglia were located within the subependymal region (Dewulf, 1937; del Rio-Hortega, 1921a,b; Wislocki & Leduc, 1952). They were also known as 'macrophage-like cells' (Oksche, 1956) or 'hypoependymal cells' (Talanti, 1926). Some authors noted that microglia in this region actively proliferated and

suggested that they were derived from ectodermal matrix (Jakob, 1927; Lewis, 1968; Metz & Spatz, 1924; Paterson et al. 1973; Pruijs, 1927; Rydberg, 1932; Schaltenbrand & Bailey, 1928). Investigations by other authors comparing the morphology of microglia with glial cells yielded contradictory results.

It is evident that these early studies, spanning over 60 years since the initial proposals of Rio-Hortega between 1919 and 1921, introduced many of the basic concepts regarding microglia with which we are familiar today. However, they were clearly limited through relying almost exclusively on morphological investigations and without recourse to the vast array of more sophisticated investigative techniques that have only been developed over the past two decades. For example, fluorescence- and light microscopic immunohistochemical detection of cell surface markers was not possible prior to the late 1970s and early 1980s, and immunogold electron microscopic studies as well as tissue culture methodologies were in their infancy during the 1960s-1970s. Moreover, these earlier investigators had at best, only a partial understanding of the lineage of mononuclear phagocytes or their functions, and a poor theoretical framework of the immune system and conditions affecting the nervous system.

Contemporary views on the morphology, phenotype, origin and functions of microglia

Much of our present understanding of microglial cell biology has stemmed from studies carried out principally in rodents. It has been estimated that microglia constitute between 5-15% of the total cellular composition of the CNS in adult rodents (Lawson et al. 1992). We now know that microglia, in addition to their role as phagocytes when activated, are capable of expressing MHC class II molecules and presenting antigen to T lymphocytes, and of production and response to a plethora of cytokines. In this respect, they represent (together with brain endothelium, perivascular cells and to a lesser extent, astrocytes) the primary line of immune defence within the adult CNS (Davis et al. 1994; Fujita et al. 1981; Ling and Wong 1993; Moore and Thanos, 1996; Perry and Gordon, 1991). Microglia can adapt to the CNS microenvironment and can change their location, morphology and functional status. This property of microglia is reflected by their differential expression of surface antigens.

Studies in rodents have shown that microglia are amoeboid, highly phagocytic, motile and immune active cells during foetal and neonatal development, capable of responding to multiple events involved with the structuring of the CNS, and formation of the neuronal-glial environment (Fujita et al. 1981; Ling and Wong 1993; Perry et al. 1985; Perry and Gordon, 1991). In the adult, microglia appear as highly ramified cells at rest, with finely-branched processes and a small oval cell body. Adult microglia occupy and monitor distinct non-overlapping territories and are evenly distributed spatially. In particular, their distribution is

not related to original sites of entry of microglial progenitors (Lawson et al. 1990). These quiescent 'resting' cells appear to downregulate both the expression of their surface molecules, and their capacity for phagocytosis, cytokine production and antigen presentation (Oehmichen et al. 1979; Perry and Gordon, 1991; Wood et al. 1979). Such recognised phenomena are in keeping with a need to protect the CNS from immune-mediated cellular damage. As a consequence however, microglia have been found notoriously difficult to visualise using conventional immunohistochemistry. Furthermore, a lack of specific microglial markers has, in the past, confounded attempts to follow the proposed developmental stages of transition between amoeboid and ramified microglial forms (Moore and Thanos, 1996).

Although microglia share a repertoire of cell surface markers found on other mononuclear phagocytes (specifically monocytes and macrophages), including CD11b/CD18 (Mac-1, CR3), CD45 (leukocyte common antigen), CD64 (FcγRI), CD68 (macrosialin, PG-M1, KP-1, EBM-11), and F4/80 (a marker of mature macrophages and monocyte-derived dendritic cells in rodents) (Perry and Gordon, 1991), these antigens are differentially expressed by subpopulations of microglia, according to their stage of development and functional activity (Hutchins et al. 1990, Rezaie and Male 1999, Rezaie et al. 1999). Histochemical staining with lectins such as *ricinus communis agglutinin-1* (RCA-1), *griffonia bandeiraea simplicifolia* isolectin-B4 (GSB4) or *lycopersicon esculentum* (tomato lectin) has proved very useful in detecting all subpopulations of microglia, including the resting forms in the adult CNS (Mannoji et al. 1986; Streit and Kreutzberg 1987; Suzuki et al. 1988).

These functional and phenotypic characteristics of microglia indicate that they belong to the mononuclear phagocyte lineage. Such cells migrate into foetal tissues prior to and during formation of the blood-brain barrier and differentiate into tissue macrophages. Evidence also considers these cells at least in part, to be derived from circulating bone marrow progenitors: initial experiments by Ling and colleagues (reviewed in Ling and Wong, 1993). It now appears that some cells derived from the bone marrow can enter the developing and adult CNS in rodents and transform to macrophages in the choroid plexus, meninges and the perivascular space (Hickey and Kimura 1988, Hickey et al. 1992; Krall et al. 1994; Flügel et al. 2001). Furthermore, electrophysiological studies have shown that microglia share a similar inward-rectifying potassium channel with a population of progenitor cells found in the bone marrow, but absent on peripheral macrophages (Kettenmann et al. 1993).

Nevertheless, the origin of parenchymal microglia is still a point for contention. While suggestions have been made that microglia arise from neuroectodermal progenitors (Fedoroff,

1995; Richardson et al. 1993), or from an intrinsic population of pluripotential haematopoietic progenitors (of the CFU-GM lineage) (Alliot et al. 1991), other views have considered these parenchymal cells to derive from mesenchymal precursors (mesodermal elements/foetal macrophages) in addition to that from circulating blood mononuclear progenitors (monocytes) that penetrate the nervous tissues in man, primarily during the latter half of intrauterine life (Rezaie, 2003; Rezaie and Male, 1999, 2002a). Despite their similarities with macrophages, microglia of the CNS differ from tissue-specific macrophages found throughout the body, by their characteristic morphology, their capacity to proliferate and by their distinct pattern of ion channels (Boucsein et al. 2000; Eder et al. 1999). They appear to 'lose' or 'downregulate' their phenotypic characteristics and enzymatic markers, typical of monocytes and macrophages, upon taking up residence within the CNS compartment (Flaris et al. 1993; Perry and Gordon, 1989). They also undergo a morphological transformation to become progressively ramified cells with the maturation of the nervous system. These characteristics are believed to be conferred on microglia as a result of their unique environment (Wilms et al. 1997). Within the adult nervous system, microglia which usually occupy distinct non-overlapping territories in their ramified conformations, are capable of rapidly 'de-differentiating' to their activated amoeboid morphological states in response to inflammatory or pathological stimuli.

Mechanisms of migration and patterns of distribution of microglia in development

The mechanisms by which microglial progenitors enter the developing CNS are currently not known. The first hypotheses to explain microglial migration during development proposed that the cells were moving towards signals from dying neurons (Ashwell 1990; Hume et al. 1983; Pearson et al. 1993; Perry 1987; Perry et al. 1985; Perry and Gordon, 1988, 1991; Schnitzer 1989). It is true that debris from dying neurons are internalised by mononuclear phagocytes and that microglia can migrate towards these dying cells (Bodeutsch and Thanos, 2000; Witting et al. 2000). However, microglial progenitors arrive in the developing nervous system, before neuronal death is prevalent (Ashwell, 1989; Ashwell et al. 1989; Diaz-Araya et al. 1995), and they are absent from certain areas of the brain where cell death is present (Milligan et al. 1991; Rakic and Zecevic, 1998).

It is well-recognised that the migration of leukocytes to particular sites of inflammation is governed by tissue-specific production of integrins, adhesion molecules, selectins and chemotactic cytokines (chemokines). Integrins are transmembrane glycoproteins that mediate cell-cell adhesion and cell-substrate interactions (Haas and Plow, 1994) **Table 1**. β1 and β2 integrins are constitutively expressed on the surface of leukocytes (in an inactive state) and integrate the intracellular cytoskeleton with the extracellular matrix (when activated).

Structurally, they are heterodimeric and consist of noncovalently associated α and β subunits, of which the β subunits are highly conserved. Integrins are constitutively expressed but do not bind to their ligand unless activated to a favourable conformational state. A consensus tripeptide RGD sequence (Arg-Gly-Asp) is necessary for integrin-ligand interactions. This tripeptide sequence is recognised by several integrins (α5β1, αIIbβ3, and most αvβ integrins). Other integrins recognise different sequences (KQAGDV, DGEA, EILDV, GPRP), and the N-terminal domains of α and β subunits combine to form a ligand-binding head on each region. Ligands for integrins include intercellular cell adhesion molecules (ICAMs), vascular cell adhesion molecules (VCAMs), CD31, complement proteins, and bacterial or viral proteins.

Table 1. The integrins, their component subunits and ligands

Integrin	β-subunit	α-subunit	Ligand
VLA family			
VLA-1	β1 (CD29)	α1 (CD49a)	Laminin, collagen
VLA-2	β1	α2 (CD49b)	Collagen, laminin
VLA-3	β1	α3 (CD49c)	Fibronectin, laminin, collagen
VLA-4	β1	α4 (CD49d)	VCAM-1, fibronectin
VLA-5	β1	α5 (CD49e)	Fibronectin
VLA-6	β1	α6 (CD49f)	Laminin
β1α7	β1	α7	Laminin
β1α8	β1	α8	
β1αv	β1	αv (CD51)	Fibronectin
LeuCAM family			
LFA-1	β2c (CD18)	αL (CD11a)	ICAM-1, ICAM-2, ICAM-3
Mac-1	β2	αM (CD11b)	ICAM-1 (ICAM-2), Fibrinogen, C3bi
P150,95	β2	αX (CD11c)	C3bi, Fibrinogen
αdβ2	β2	αd	ICAM-3
Cytoadhesin family	β3 (CD61)		
CD41a		αIib (CD41)	Fibrinogen, fibronectin, vitronectin, vWf
Vitronectin receptor		αv (CD51)	Vitronectin, fibrinogen, vWf, laminin, thrombospondin, fibronectin, PECAM (CD31)
β4α6	β4 (CD104)	α6 (CD49f)	Laminin
β5αv	β5	αv (CD51)	Vitronectin, fibronectin
β6αv	β6	αv (CD51)	Fibronectin
β7α4, LPAM-1	β7	α4 (CD49d)	Fibronectin, VCAM-1, MadCAM-1

Integrins are recognised as important in many processes including inflammation, angiogenesis, cellular gowth and differentiation, and for their involvement in directing cell migration and neurite outgrowth during development (DeSimone, 1994). **Table 1** lists some important integrins and their ligands. Cellular adhesion molecules including ICAM-1, ICAM-2, VCAM-1 and PECAM, which act as ligands for integrins, are members of the immunoglobulin superfamily. Selectins (P-, S- and L-) are a family of calcium-dependent carbohydrate-binding proteins that enable initial adhesion of leukocytes to the wall of blood vessels during inflammation (Bevilacqua 1993). They bind to sialylated and fucosylated

carbohydrate ligands and permit leukocytes to roll along the endothelial lumenal surface, prior to anchoring at sites downstream. Thus, the role of selectins is restricted to directing leukocyte interactions with vascular endothelium.

The binding of integrins on leukocytes to cell adhesion molecules (CAMs), either constitutively expressed or induced on the endothelium, lead to interactions with the cytoskeleton that allow the migration of cells into lymphoid, non-lymphoid and inflamed tissues (Male et al. 1994). This process consists of at least two events, of which the first directs migration of circulating cells across vascular endothelium, the second involves localisation to particular zones within tissues. By analogy with the migration of leukocytes into other tissues, the expression of certain adhesion molecules during embryonic development and in the early postnatal period could play an important role in the recruitment of haematogenous progenitors of microglial cells. If these progenitors do indeed derive from the circulation, at least three steps could be potentially involved in their migration through cerebral endothelium: (i) circulating cells are slowed down by interaction between their carbohydrate residues and selectins (e.g. E-, L- or P-selectin) expressed on the lumenal aspect of the cerebral endothelium, (ii) arrested cells may be triggered by cytokines (such as MCP-1 or IL-8) held on the endothelial surface, and/or through homotypic interaction between PECAM-1 (CD31) on the endothelium and leukocyte surfaces, (iii) triggering causes activation of leukocyte integrins which subsequently interact with adhesion molecules (e.g. ICAM-1, ICAM-2, VCAM-1) expressed on vascular endothelium (Ebnet et al. 1996; Male et al. 1994). According to this scenario, it is possible that some populations of microglial progenitors use combinations of well-defined adhesion molecules to enter the developing CNS. Alternatively the process may be controlled by adhesion molecule(s) that are expressed at particular times on CNS endothelium during development.

Table 2. Some important chemokines and their receptors

Chemokine Family	Receptor	Ligand
CC chemokines	CCR1	RANTES, MIP-1α, MCP-2, MCP-3
	CCR2	MCP-1, MCP-2, MCP-3, MCP-4
	CCR3	Eotaxin, RANTES, MCP-3, MCP-4
	CCR4	RANTES, MIP-1α, MCP-1
	CCR5	RANTES, MIP-1α, MIP-1β
CXC chemokines	CXCR1	IL-8
	CXCR2	IL-8, GRO-α/β/γ, NAP-2, ENA78, GCP-2
	CXCR3	IP-10, Mig
	CXCR4	SDF-1
CX_3C chemokine	CX_3CR1	Fractalkine

Equally important is the role of chemokines in directing this migratory phenomenon (refer to Rezaie and Male, 1999; Rezaie et al. 2002a). Chemokines are a group of chemotactic cytokines that activate leukocytes and are essential for directing the migration of these cells under normal circumstances, and particularly in inflammation (Bacon and Oppenheim, 1998; Ransohoff and Tani, 1998; Sozzani et al. 1996; Zlotnik and Yoshie 2000). More than 75 chemokines have now been described, the majority of which belong to two distinct families: CXC (or α-) chemokines and CC (or β-) chemokines **Table 2**. They can be distinguished according to the position of cysteine residues forming disulphide bonds that are either adjacent (CC) or separated by an amino acid (CXC) in the N-terminal region. Their corresponding genes are located on chromosomes 4 (CXC) and 17 (CC) respectively. All are secreted, with the exception of fractalkine (a CX_3C chemokine), which has both transmembrane and cytoplasmic domains. The β-chemokines are potent chemoattractants for monocytes (but not neutrophils) *in vitro*. With few exceptions (Mig and PF4), α-chemokines preferentially chemoattract neutrophils. CXC and CC chemokines can form symmetrical homodimers as quaternary structures. At high concentrations (>100μM), they adopt a dimeric form. At physiologically active concentrations (>100μM) they generally occur as monomers, and chemokines elicit biological activities at nanomolar concentrations *in vivo* (Paolini et al. 1994). Fractalkine (also referred to as neurotactin) is constitutively expressed at high levels within the normal adult CNS and upregulated in pathology (Bazan et al. 1997; Harrison et al. 1998; Imai et al. 1997; Maciejewski-Lenoir et al. 1999; Nishiyori et al. 1998).

By sequence comparison, fractalkine is most closely related to the β-chemokine MCP-1 (Pan et al. 1997). Chemokines have overlapping functions and display affinity for more than one receptor. Their receptors are coupled to G-proteins via the intracytoplasmic and carboxy terminals. These receptors possess seven transmembrane domains and belong to the serpentine superfamily of receptors, 18 of which have been identified (CCR1-11, CXCR1-5, XCR1, CX_3CR1: Murphy et al. 2000 for review). Competition binding and cross-desensitisation studies have shown CCR2, CCR4 and CCR5 to be expressed on human monocytes. Both CCR5 and CXCR4 have been detected on perivascular mononuclear cells of the brain and on macrophages. CXCR4 has further been reported on subpopulations of neurons *in situ* and in culture.

Interest in the chemokines and their receptors has been at the heart of research into neuroinflammation over the past few years (Bacon and Harrison 2000; Bacon and Oppenheim 1998; Glabinski and Ransohoff, 1999; Hesselgesser and Hork, 1999; Leung et al. 1997; Locati and Murphy, 1999; Meda et al. 1996; Mennicken et al. 1999; Sanders et al. 1998; Woodroofe et al. 1999; Xia and Hyman, 1999; Zhang et al. 2000). Chemokines now appear to possess

multiple biological activities in addition to their known roles in mobilising inflammatory cells. These include roles in development, growth and migration of cells, angiogenesis, haematopoiesis, phagocytosis, free radical production, apoptosis, T-cell activation, neoplasia, inflammatory regulation in response to injury, wound healing, tissue repair and macrophage recruitment, as well as interactions with pathogens including viruses (Bacon and Harrison, 2000; Baggiolini et al. 1997; DiPietro et al. 1995; Engelhardt et al. 1998; Gibran et al. 1997; Glabinski and Ransohoff, 1999; Hesselgesser and Horuk, 1999; Jackman et al. 2000; Locati and Murphy, 1999; Schall and Bacon, 1994).

A wide variety of cells produce chemokines. Endothelial cells in particular, not only synthesise chemokines, but possess the ability to transport these molecules from the ablumenal to the lumenal surface. The leukocyte migratory response is co-ordinated with the expression of relevant adhesion molecules, cytokines and proteases (Proost et al. 1996). Chemokines appear to be particularly important for activating leukocytes and inducing the transition from rolling to firm adhesion to the endothelium. Cells are likely to encounter several chemokine gradients as they move throughout tissues, and there is further evidence for the presence of hierarchies of chemokines that direct migration to particular sites (Foxman et al. 1997). Those with a higher specific activity are active even at low concentrations at invariably greater distances from the site of production. Less potent chemokines require a higher concentration and are active closer to the site of production. By example, in rat lungs, RANTES and MCP-1 (as well as GM-CSF) produced by alveolar epithelial cells, control the distribution of alveolar macrophages through the creation of local chemotactic gradients (O'Brien et al. 1998). Diapedesis and migration towards a site of infection is similarly controlled by chemokine gradients (Schall, 1994; Miller and Krangel, 1992). The binding of chemokines to extracellular matrix components and cell surface molecules (for example via heparan sulphate proteoglycans) can confine or immobilise chemokines to the sites of production and thus help to establish hierarchical gradients of chemokines that direct site-specific migration of leukocytes (Baggiolini et al. 1997; Cuff et al. 2000; Foxman et al. 1997; Hesselgesser and Horuk, 1999; Locati and Murphy, 1999; O'Brien et al. 1998; Rot, 1992; Tanaka et al. 1993; Webb et al. 1993; Witt and Lander, 1994).

There are expanding roles for chemokines in the development, and signalling of embryogenic events. These include the positioning of cells within appropriate subcompartments of tissues (e.g. foetal liver, bone marrow and thymus), lineage development, the recruitment of bone marrow prgenitors to foetal tissues (e.g. lungs, kidney and epidermis), and the patterning and development of organs (Forster et al. 1996; Gunn et al. 1999; Jazin et al. 1997; Jotereau et al. 1997; Kukita et al. 1997; von Luettichau et al. 1996; Ma et al. 1999; McGrath et al. 1999;

Nakamura et al. 1995; O'Brien et al. 1998; Volejnikova et al. 1997; Votta et al. 2000; Wilkinson et al. 1999). Recent data suggest additional roles for chemokines in the maintenance of CNS homeostasis and neuronal patterning during development (Hesselgesser and Horuk, 1999; Horuk 1998; Ma et al. 1998; Mennicken et al. 1999; Zou et al. 1998). Specifically, microglia are known to express chemokine receptors (CCR1, CCR2, CCR3. CCR5, CCR10, CXCR4, CX3C) and migrate in response to a variety of chemokines (MIP-1α, MIP-1β, MIP-3, MCP1-5, RANTES, SDF-1 and fractalkine) (Cross and Woodroofe, 1999). It is therefore possible that the migration of microglial progenitors to the CNS during development, is initiated by intracerebral gradients of chemotactic agents such as chemokines.

Why investigate colonisation of the developing CNS by microglia?

Studies in rodents have shown that microglia colonise the nervous system in the period just before birth, but mainly throughout the postnatal period (Miyake et al. 1984; Perry et al. 1985; Dalmau et al. 1997). Microglial resting morphology, growth and adult numbers are attained within several days following birth, and these cells have been shown to distribute within the first two postnatal weeks (Perry, 1987-1997). As a result, the postnatal period has been the primary focus for studying the differentiation of amoeboid microglia to ramified cells, a transformation which is gradually accompanied by the disappearance of amoeboid cells, which are no longer detectable in the mature brain (Ling and Wong, 1993). Our knowledge of these perinatal and postnatal events in rodents is significant. However, by comparison, less is known about the routes and mechanisms of migration during the foetal period in these species. Considerably less is known about such events in the developing human nervous system.

Elucidating the phasing, patterns and mechanisms of microglial colonisation of the human CNS *in situ* are not only important from a developmental point of view. Knowledge of these events will be of direct significance to studies that address somatic gene therapy of human disease (for example the hereditary lipidoses), in which microglial progenitors may be used as potential gene carriers into the CNS. Transplantation studies indicate that some progenitor populations for microglia exist within umbilical cord blood (Krivit et al. 1995, 1998; Unger et al. 1993) and that genetic defects can be partly corrected by the transplantation of bone marrow or umbilical cord blood. Conceivably, microglial progenitors can be engineered to carry normal genes into the CNS. The main underlying purpose for this investigation will therefore be to understand the timing and mechanisms by which the human central nervous system is colonised by microglia. This information is critical from a clinical approach, in its potential application to gene therapy for a number of paediatric neurological disorders, including the hereditary lipidoses. The mouse has emerged over the last two decades, as a most useful experimental model for a wide variety of neurological disorders. In order to

consider practical approaches towards this aim in the clinic, one would also need to investigate colonisation of the murine CNS *in situ* during the *foetal* period prior to birth, if this species is to be used as a valid experimental model that will faithfully replicate both disease and its treatment stratagem.

Microglia in tissue culture

The morphological transformations and motile behaviour of microglia have been best characterised from studies in tissue culture. Del Rio-Hortega's original descriptions of microglia between 1919 and 1921 were soon followed by the first accounts of these cells in culture, dating back to 1925. The transformation of various morphological 'transitory' states of microglia were observed in these culture studies, and there was general agreement at the time that similar changes took place *in vivo*: the amoeboid and pseudopodic cells were recognised as the motile, migratory forms, while the branched cells were more stable forms (Costero 1930a, 1930b, 1930c, 1931; Dunning and Furth 1935, del Rio-Hortega, 1932; Kershman 1939; Penfield, 1928, Wells and Carmichael 1930). We now know that the procedure of isolating microglia inadvertently results in the activation of these cells, characterised by their amoeboid morphology, expression of adhesion molecules and production of large amounts of free radicals (Heppner et al. 1998). However, the deactivation and accompanying ramification of microglia are precipitated by cellular interactions within the CNS microenvironment. Microglial cultures have been produced either pure (i.e. >90%) or in co-culture with astrocytes. Specifically, experiments *in vitro* have emphasised the importance of astrocytes in regulating microglial transformation (Noble et al. 1994; Schmidtmayer et al. 1994; Sievers et al. 1994). Although the majority of the more recent *in vitro* studies have concentrated on microglia isolated from the rodent CNS, methods for isolating human foetal microglia have also been developed (Hassan et al. 1991; Lauro et al. 1997; Lee et al. 1992; Peudenier et al. 1991), and several reports have highlighted the differentiation of human foetal microglia in co-culture with astrocytes (Hassan et al. 1991; Lauro et al. 1995; Lee et al. 1994; Liu et al. 1994). In such preparations, ramified cells are usually located above or under astrocyte monolayers, whereas amoeboid cells occur more frequently overlying astrocytes (Tanaka et al. 1999). Ramified microglia have also been described in slice preparations (Hailer et al. 1996) and dissociated cultures obtained from rodent CNS (Booth and Thomas, 1991). Notably, these cells possess a high level of pinocytic activity (determined from sequestration of dyes in pinocytic vesicles), but little or no phagocytic activity (Ransom and Thomas, 1991; Ward et al. 1991). A few studies have investigated microglia using time-lapse video microscopy (Booth and Thomas, 1991; Brockhaus et al. 1993; Nolte et al. 1996; Schiefer et al. 1999; Stence et al. 2001; Thomas 1990; Turley et al. 1994; Ward et al. 1991). Only two of these reports have directly analysed

microglial motility using cellular recordings in relation to morphology, and these were carried out in CNS slice preparations (Brockhaus et al. 1993; Stence et al. 2001). The main focus for these studies has been directed at the deramification and activation of microglia following culture.

Given the regulatory influence of astrocytes on microglial morphology and function which are indicated in these *in vitro* studies, it is important to determine the temporal and spatial relationship between these two cell types *in situ* in the developing nervous system and the interaction between these cell types in tissue culture with respect to the differentiation of human foetal microglia, and their production of and response to chemokines that may be involved in directing microglial dispersion throughout the nervous system during development.

Aims and Objectives

The main purpose of this investigation was to understand the temporo-spatial phasing and mechanisms by which the nervous system is colonised by microglial progenitors. These data are of particular application to potential therapies for a number of paediatric disorders affecting the brain, including hereditary lipidoses (Niemann-Pick disease, Gaucher's disease, Krabbe disease and metachromatic leukodystrophy) (Scriver et al. 1989), which are caused by single enzyme deficiencies that may lead to the accumulation of lipids within mononuclear phagocytes and subsequently to progressive neuropathological damage of variable severity. These defects may be partly corrected by transplantation of normal bone marrow or cord blood, rich in haematopoietic progenitors.

Therefore the objectives of this investigation were to determine (i) the phasing and patterns of microglial colonisation of the developing human and murine nervous system, (ii) mechanisms by which microglial progenitors colonise the brain and spinal cord during foetal development including their interaction with astrocytes, and (iii) to investigate possible signals that may direct the migration of these precursor cells and affect their distribution primarily in the developing human nervous system.

Chapter II MICROGLIA IN THE DEVELOPING HUMAN CNS

In this Chapter we shall first determine the location, morphology and phenotype of microglia in the human foetal brain and spinal cord, and progress to the expression of potential signals driving the recruitment and differentiation of microglial progenitors: (i) vascular adhesion molecules, (ii) chemokines, and (iii) regional apoptosis. Expression of defined adhesion molecules and chemokines and the distribution of astrocytes will be examined for correlation with areas colonised by foetal microglia. The following questions will be addressed:

- What are the phases of colonisation and regional distribution patterns of microglial progenitors within the human foetal brain and spinal cord during the second trimester?
- It is known that microglia in tissue culture interact with astrocytes and that signals received from these cells can cause microglia to differentiate. Does a temporo-spatial relationship hold between microglial progenitors and astrocytes *in situ*, within human foetal CNS?
- Are microglial progenitors recruited via blood vessels, and which defined adhesion molecules are expressed on cerebral vessels as potential signals for recruiting these cells?
- Which chemokines are expressed within the human foetal brain during the second trimester? How are they distributed, and do microglial progenitors possess the corresponding chemokine receptors that they may use to migrate towards sources of these chemoattractants?
- Do microglial progenitors specifically accumulate within areas undergoing developmentally-regulated apoptotic cell death?

MATERIALS & METHODS

Human tissue samples

Frozen and formalin-fixed paraffin-embedded CNS tissues from foetal and adult human postmortem cases were obtained for this study from the MRC Brain Bank, Institute of Psychiatry, King's College London (courtesy of Dr. Nigel Cairns and Nadeem Khan, co-ordinators), and the MRC Tissue Bank, Hammersmith Hospital, London, UK (courtesy of Dr. L. Wong, Honorary Consultant and Director). **Tables 3-5.** All materials were acquired with prior informed consent and approval of the local ethical committees of the Institute of Psychiatry and the Bethlem, Maudlsey and King's Healthcare NHS Trusts. Gestational age of foetal material is given in weeks (gestational weeks or GW) and was estimated according to the following criteria in order of preference: anatomically by crown-to-rump length, head circumference, foot length, hand measurements, or calculated from the last menstrual period. There was no evidence of intracranial pathology or germinal layer haemorrhage in foetal material included in these studies. Where indicated, tissue had previously been snap frozen by contact with a brass plate at -70°C and stored at this temperature until use. Frozen sections 20-60μm thick, were cut from each block of tissue onto silane-coated slides using a cryostat and stored at -70°C for histological analysis. Cryostat sections from adult human brain (pathological and non-pathological tissues), a normal infant brain and paraffin-embedded sections of human tonsil were used as controls. Serial paraffin-embedded sections (7-10μm thick) taken from five normal foetal brains (19-23GW) were also used for this study. In addition, a further four paraffin-embedded, mercuric-chloride fixed tissue blocks from postnatal human CNS [NP106-83 (1day); NP101-83 (6 weeks): NP3-83 (3 months); NP55-83 (5 months)] were obtained from Dr. Brian Harding, Department of Histopathology, Great Ormonds Street Childrens Hospital NHS Trust, London. Sections from these cases were treated with Lugol's iodine (10 minutes) and sodium hyposulphite (2 minutes) in order to remove the residual mercuric precipitate, prior to use.

Table 3. Details of human foetal brain tissues used in this investigation

Gender	Gestational Age	PM delay	Cause of death	Pathologial Diagnosis	Block
M	12-13	6	Prostaglandin-induced termination	Normal brain	coronal paraffin
M	13-14	30	Spontaneous miscarriage	Normal brain	coronal paraffin
M	14	>72	Uterine rupture	Normal brain	coronal paraffin
F	16	27	Spontaneous miscarriage	Normal brain	coronal frozen
M	17*	62	Spontaneous miscarriage	Normal brain	coronal frozen
M	18**	24	Spontaneous miscarriage	Normal brain	coronal frozen
F	18**	14	Spontaneous miscarriage	Normal brain	coronal frozen
M	19	16	Prostaglandin-induced termination	Normal brain	horizontal frozen
F	19-20	NA	Prostaglandin-induced termination	Normal brain	sagittal frozen
F	20*	36	Spontaneous miscarriage	Normal brain	coronal frozen
F	20	36	Prostaglandin-induced termination	Normal brain	coronal frozen
M	20	32	Spontaneous miscarriage	Normal brain	horizontal frozen
F	22	32	Spontaneous miscarriage	Normal brain	horizontal frozen
M	23	48	Spontaneous miscarriage	Normal brain	coronal frozen
F	23*	>72	Premature delivery	Normal brain	coronal frozen
F	23*	42	Prostaglandin-induced termination	Normal brain	coronal frozen
M	19	63	Induced termination	Trisomy 21	coronal frozen
M	21	6	Induced termination	Trisomy 21	coronal frozen
M	23	6	Induced termination	Trisomy 21	coronal frozen
M	33-36	72	Induced delivery- stillborn	White matter leukomalacia, global ischaemia/hypoxia	coronal paraffin
M	8 weeks postnatal (32+8)	---	Hydrocephalus secondary to subacute encephalitis and ventriculitis	Acute-on-chronic ventriculo-encephalitis (suspected toxoplasmosis, not confirmed)	coronal paraffin

[*] Frozen by immersion in isopentane in liquid nitrogen;
[**] Frozen samples: rapidly frozen by contact with a brass plate kept at -70^0C
[---] Indicates information not available
Where cases of spontaneous miscarriage are recorded, no foetal congenital abnormalities were present, unless otherwise stated.
Where 'normal brain is indicated', there was no evidence of ischaemic/hypoxic injury, or haemorrhage either within the germinal matrix or associated with the meninges.
Gestational age is given in weeks, and was determined according to a number of parameters including crown-rump measurement, anatomically by foot length, or by history of the last menstrual period

Table 4. Details of human foetal spinal cord tissues used in this investigation

Specimen No.	Menstrual Age/weeks	Conception Age/weeks	Orientation
*13853	9.7	7-8	longitudinal
14065	10.4	8-9	transverse
13616	10.8	8-9	transverse
13580	11.0	9	transverse
13374	11.5	9-10	transverse
13400	12.4	10-11	transverse
14167	12.9	10-11	transverse
13945	14.6	12-13	transverse
13966	16.3	14-15	transverse

[*] menstrual age determined anatomically by hand measurements;
in all other cases, menstrual age was determined by a number of parameters including crown-rump measurement, anatomically by foot length, or by history of the last menstrual period. Conception age was estimated as menstrual age less two weeks. All samples were from thoraco-lumbar regions.

Table 5. Details of adult human tissue samples used in this investigation

Gender	Age/years	PM Delay/hrs	Cause of Death	Pathological Diagnosis
F	63	>24	myocardial infarction	normal aged brain
F	71	30	pulmonary embolism	normal aged brain
M	83	>24	myocardial infarction	normal aged brain
F	80	9	bronchopneumonia, dementia	Alzheimer's disease
F	81	31	bronchopneumonia	Alzheimer's disease
M	73	6	bronchopneumonia	diffuse Lewy body disease
M	78	7	bronchopneumonia	diffuse Lewy body disease
M	41	<24	pulmonary infection	HIV encephalitis and CMVI
M	48	---	AIDS	HIV encephalitis (subacute)
M	53	<48	terminal dementia, MND	frontal-type dementia, MND
F	69	24	motor neuron disease	motor neurone disease
M	47	7	bronchopneumonia	multiple sclerosis
F	54	32	pulmonary embolism	multiple sclerosis
F	63	92	bronchopneumonia	normal spinal cord
F	64	60	carcinoma of bladder	normal spinal cord
M	76	41	bronchopneumonia	normal spinal cord
M	80	6	carcinoma of prostate	normal spinal cord
M	86	70	peritonitis	normal spinal cord

Abbreviations: AIDS: acquired immunodeficiency syndrome;
HIV: human immunodeficiency virus;
CMVI: cytomegalovirus infection;
MND: motor neurone disease.

Protocol 1. IMMUNOHISTOCHEMISTRY

A modified streptavidin-biotin-HRP (ABC) method (Dako, UK) was used for single and dual-label immunohistochemistry. A list of all immunoreagents used and their dilutions is given in **Table 6** and **Table 7**. All antibodies were routinely tested to determine optimal dilutions. Frozen sections were thawed at room temperature and air-dried for two hours, prior to immersion in a solution of methanol containing 2.5% of a 30% hydrogen peroxide solution for 90 minutes. Sections embedded in paraffin wax were first dewaxed in xylene, rehydrated in 100% alcohol (2 x 5 minutes) prior to immersion in the methanol/hydrogen peroxide solution. Sections were next rinsed with deionised water, then washed twice in phosphate buffered saline solution (PBS, pH7.6) (comprising 8g NaCl [0.14M], 0.2g KCl [2.7mM], 0.2g KH_2PO_4 [1.5mM] and 1.15g Na_2HPO4 [8.1mM] made up in 1 litre of deionised water). They were preincubated with Hanks Balanced Saline Solution (HBSS, Sigma UK) containing 1% of each of the following: [1M] $MgCl_2$, [1M] $CaCl_2$ Tween 20, and bovine serum albumin (BSA, Sigma UK) for 2 hours at room temperature and pressure (rtp). The solution was drained off the slides and they were incubated for a further 2 hours in normal serum (rabbit serum for monoclonal antibodies raised in mice, rabbit serum for antibodies raised in goats, swine serum for polyclonal antibodies raised in rabbits, DAKO, UK) diluted 1:10 with PBS to block non-specific binding. The solution was drained off the slides and sections were incubated overnight either at 4°C or at room temperature with the primary antibody made up in 1:100 dilution of the respective normal serum. Sections were then washed twice in PBS and reacted with biotinylated rabbit anti-mouse IgG (for mouse monoclonal antibodies), rabbit anti-goat IgG (for goat polyclonal antibodies), or swine anti-rabbit IgG (for rabbit polyclonal antibodies) (DAKO, UK), diluted 1:100-200 in PBS, for 2 hours at room temperature. Two subsequent washes with PBS were followed by a 2 hour incubation with streptavidin-biotin-horseradish peroxidase complex (ABC-HRP kit, DAKO UK) prepared 30 minutes prior to application, and a further two washes with PBS. 3,3`-diaminobenzidine tetrachloride (DAB, 0.25 or 0.5mg/ml made up in PBS) and 0.05% hydrogen peroxide were used as substrate for the HRP, to visualise the bound primary antibody (brown reaction product).

Table 6. Antibodies used for immunohistochemistry to human determinants, and lectins for histochemistry

	Clone	Reactivity	Dilution	Source
Monoclonal mouse antibodies				
CD11b [Mac-1, C3bi receptor, αM integrin]	44	Macrophages, monocytes, granulocuytes, NK cells	1:5-20	Cambridge Bioscience, UK
CD11b [Mac-1, C3bi receptor, αM integrin]	2LPM19c	Macrophages, monocytes, granulocuytes, NK cells	1:5-20	Dako, UK
CD45 [LCA]	Mem 55	Bone-marrow-derived cells, including macrophages	1:5-20	Cambridge Bioscience, UK
CD45 [LCA]	2B11+ PD7/26	Bone-marrow-derived cells, including macrophages	1:10-20	Dako, UK
CD64 [FcγR1]	10.1	Monocytes and macrophages	1:5-10	Serotec Ltd, UK
CD68 [macrosialin]	PG-M1	Monocytes and macrophages	1:100	Dako, UK
CD68 [macrosialin]	KP-1	Monocytes and macrphages	1:100	Dako, UK
HAM-56	MA 935	Macrophages and endothelial cells	1:5-20	Cambridge Bioscience, UK
CD31 [PECAM]	HC1/6	Monocytes, platelets, endothelial cells, tissue macrophages	1:100	Serotec, UK
CD54 [ICAM-1]	84H10	Lymphocytes, monocytes, granulocytes, activated endothelium	1:100	Serotec, UK
CD102 [ICAM-2]	B-T1	Endothelial cells	1:100	Serotec, UK
CD62E [E-selectin]	1.2B6	Leukocytes, monocytes, lymphocytes, neutrophils, endothelium	1:100	Serotec, UK
CD62P [P-selectin]	AK-6	Leukocytes, monocytes, lymphocytes, neutrophils, endothelium	1:100	Serotec, UK
CD106 [VCAM-1]	1.G11B1	Activated endothelial cells, some tissue macrophages, bone marrow fibroblasts and myoblasts	1:100	Serotec, UK
Vimentin (57KD intermediate filament)	Vim 3B4	Mesenchymal precursors, lymphoid cells, smooth muscle cells, fibroblasts, spongioblasts (including astrocyte progenitors)	1:100	Dako, UK
PCNA (cyclin)	19 A2	Cell cycle regulated protein, appearing in G_1 and throughout S-phase of proliferating cells	1:100	Serotec, UK
Polyclonal antibodies				
Glial fibrillary acidic protein [GFAP] (rabbit)	---	Astrocytes, glioblast progenitors (including radial glia)	1:500	Dako, UK
CD62P [P-selectin] (rabbit)	---	Leukocytes, monocytes, lymphocytes, neutrophils, endothelium	1:100	Cambridge Bioscience, UK
Laminin (rabbit)		Present on a wide variety of cell types and the ECM, particularly restricted to endothelial cells during development	1:100	Serotec, UK
Biotinylated lectins				
Ricinus communis agglutinin-1 [RCA-1/RCA_{120}]	---	Macrophages, monocytes, endothelial cells	1:250-500	Vector Labs., UK
Griffonia bandeiraea simplicifolia isolectin B4 [GSB4]	---	Macrophages, monocytes, endothelial cells	1:100-500	Vector Labs., UK
Lycopersicon esculentum [Tomato lectin]	---	Macrophages, monocytes, endothelial cells	1:100-250	Vector Labs., UK

Table 7. Antibodies directed against human chemokines and chemokine receptors

	Clone/lot	Dilution	Source
mAbs to human chemokines			
MIP-1α	Cat 500-M74	1:100	PeproTech EC Ltd, UK
MIP-1α	11A3	1:500	Srotec, UK
MCP-1/MCAF[a]	Lot 056L022	1:100	PeproTech EC Ltd., UK
MCP-1/MCAF	10F7	1:100	Serotec, UK
MCP-3	36320.11	1:100	R & D systems, Europe
IL-8	Lot 026Y031	1:500	PeproTech EC Ltd., UK
pAbs to human chemokines			
MIP-1β (rabbit)	Lot 047E361	1:10-1000	PeproTech EC Ltd. UK
RANTES (rabbit)	Cat 500-P78	1:500	PeproTech EC Ltd. UK
IP-10 (rabbit)	Lot 017E391	1:1000	PeproTech EC Ltd. UK
SDF-1/PBSF (goat)	Lot AKU02	1:10-100	R & D systems, Europe
Fractalkine (rabbit)	Cat 500-P98	1:1000	PeproTech EC Ltd. UK
mAbs to human chemokine receptors			
CCR2[b]	48607.121	1:100-250	R & D systems, Europe
CCR5	2D7	1:20-50	Becton Dickinson Ltd., UK
CCR5[c]	45549.111	1:20	R & D systems, Europe
CXCR1	42705.111	1:25	R & D systems, Europe
CXCR4	12G5	1:20-50	Becton Dickinson Ltd., UK
CXCR4[d]	44716.111	1:50	R & D systems, Europe

mAb (monoclonal antibodies); *pAb* (polyclonal antibody)
[a]no cross-reactivity with other MCP or structutally related chemokine epitopes
[b]no cross-reactivity with CCR5
[c]no cross-reactivity with CCR1/2/3
[d]no cross-reactivity with other chemokine receptors

Following development of the first immune bound enzyme complex with DAB, sections were rinsed with deionised water, then preblocked with the appropriate normal serum (swine serum for polyclonal antibodies raised in rabbits), incubated with primary and secondary antibodies, and ABC-HRP according to the protocol described. The reaction was visualised using VIP (Vector Laboratories, UK) to obtain a violet reaction product. Alternatively, after the secondary antibody incubation step, sections were incubated with streptavidin-biotin complex bound to alkaline phosphatase, and the activity of this enzyme was demonstrated by incubating sections with Vector Red substrate (Vector Laboratories, UK) or overnight (in the dark, at room temperature) in nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) substrate (containing 1μl/ml levamisole to inhibit endogenous alkaline phosphatase activity, Vector Laboratories, UK). Sections were washed under running tap water, nuclei were lighly counterstained with haematoxylin or methyl green, and sections were differentiated, dehydrated in a series of graded alcohols, cleared in Xylene and mounted using Permount, or DPX (VWR-Merck International, UK) for light microscopic analysis. For methyl green counterstaining, the solution was first preheated to 60°C, slides were incubated for 1-5 minutes, then rinsed for 1 minute with deionised water. Excess water was drained and

slides were dipped 5-10 times in acetone containing 0.05% (v/v) acetic acid. Sections were dehydrated immediately in 95% alcohol, and two changes of 100% alcohol. cleared in xylene and mounted. Alternatively, the acetone/acetic acid step was eliminated and slides processed as per usual in graded alcohols (70-100%). All single and double-immunostaining procedures were performed on batches of slides on the same occasion to limit randomisation of intensity or conditions. Adjacent sections were stained with haematoxylin and eosin, to identify structural morphology.

For optimising the immunoreactive protocol, the original diluent buffer routinely used within the Departmental protocols (Tris-buffered saline solution, pH7.6: TBS containing 60.45g Trizma base, 85g NaCl per liter of water to which is added 32mls of concentrated HCl, and pH adjusted to 7.6), was replaced with PBS, as this buffer appeared to produce consistently less background immunoreactivity. Likewise Triton-X100 detergent used routinely for permeabilising the tissues was substituted with Tween 20, as this similarly reduced background immunoreactivity, particularly on frozen tissue sections for dual-label immunohistochemistry. Negative control sections were included, where the primary antibodies were omitted or replaced with control IgG (isotype mouse, rabbit or goat IgG, Vector Laboratories, UK).

Protocol 2. LECTIN HISTOCHEMISTRY

Background: Lectins were discovered in the 19th century, when it was found that extracts from certain plants could agglutinate red blood cells (Liener et al. 1986). The term 'lectin' was given to agglutinins that could discriminate between red blood cell types. This term now includes proteins that can bind to defined sugars (glycoconjugates) in plants, microorganisms, viruses and animals. The majority of lectins consist of multimeric non-covalently associated subunits. Each lectin demonstrates preferential binding to a particular carbohydrate substrate, allowing discrimination between mannose, glucose, lactose and galactose residues **Table 8**. Some of these lectins require the particular sugar to be located at a terminal (non-reducing) position in the oligosaccharide, whereas others can bind within the oligosaccharide chain. The α- and β-isomeric structure of sugars is a separate factor that determines lectin binding. Practically all biological membranes and cell walls contain glycoconjugates that can be analysed using lectins. Occasionally, lectins have been found to induce mitosis in cells which do not normally divide, although the exact mechanism underlying this is unclear (Liener et al. 1986).

Table 8. Specifcity of some commonly used lectins

Sugar recognised	Lectin
Fucose	AAL, LTL, UEA I
Glucose	Con A, LCA, PSA
Galactose	ACL, ECL, EEL, GSL I, **GSL I-B_4**, MAL I, PNA, RCA_{60}, **RCA_{120}**, SBA
Mannose	Con A, GNL, HHL, LCA, NPL, PSA
N-acetylglucosamine	DSL, **LEL**, STL, WGA
N-acetylgalactosamine	BPL, DBA, GSL I, MPL, PTL, SJA, RCA_{60}, **RCA_{120}**, SBA, VVA, WFA
Sialic acid	MAL II, SNA

Abbreviations:
AAL: *Aleuria aurantia*; ACL: *Amaranthus caudatus;* PNA: *Arachis hypogea* (peanut lectin); Jacalin: *Artocarpus integrifolia* (Jackfruit lectin); BPL: *Bauinia purpurea alba* (Camels foot tree lectin); ConA: *Canavalia ensiformis* (Jack bean lectin); DSL: *Datura stramonium* (Thorn apple/jimson weed lectin); DBA: *Dolichos biflorus* (Horse gram lectin); ECL: *Erythrina crystagalli* (coral tree lectin); EEL: *Euonymus europaeus* (spindle tree lectin); GNL: *Galanthus nivalis* (snowdrop lectin); SBA: *glycine max* (soybean lectin); GSL: *Griffonia (Bandeiraea) simplicifolia*; HHL: *Hippeastrum hybrid* (Amaryllis lectin); LCA: *Lens culinaris* (lentil lectin); LTL: *Lotus tetragonolobus* (winged pea lectin)/*Tetragonolobus purpurea* (asparagus pea lectin); LEL: *Lycopersicon esculentum* (tomato lectin); MAL: *Maackia amurensis*; MPL: *Maclura pomifera* (Osage orange lectin); NPL: *Narcissus pseudonarcissus* (daffodil lectin); PSA: *Pisum sativum*; PTL: *Psophocarpus tetragonolobus* (Winged bean lectin); RCA: *Ricinus communis* (castor bean lectin); SNA: *Sambucus nigra* (elderberry lectin); STL: *Solanum tuberosum* (Potato lectin); SJA: *Sophora japonica* (Japanese pagoda tree lectin); WGA: *Trticum vulgaris* (wheat germ lectin); UEA: *Ulex europaeus* (Furze gorse lectin); VVA: *Vicia villosa* (Hairy Vetch lectin); *Wisteria floribunda* (Japanese Wisteria lectin).

Lectins such as RCA-1, GSB-4, mistletoe and tomato lectin have gained considerable popularity for their use in reliably detecting microglia, irrespective of their morphological or immunophenotypical states (Hewicker-trautwein et al. 1996; Rezaie et al. 1997, 1999; Suzuki et al. 1988; Wierzba-Bobrowicz et al. 1995). They are especially valuable for studies on macrophage precursors and foetal and embryonic macrophages, since they identify these cells when only a limited number of anti-macrophage antibody markers are useful, and before cells can be distinguished by their high lysosomal enzyme activities (Kaur et al. 1990; Sorokin et al. 1994). By contrast, immunohistochemistry appears to be more varied in identifying microglia, due to the differential expression of surface antigens (refer to Chapter V). These lectins specifically stain lactose and galactose (RCA-1, GSB4) and N-acetylglucosamine (tomato lectin) residues on the surface of macrophages and microglia as a well as a large proportion of cerebral vascular endothelium (Mannoji et al. 1997). Expression of surface galactopyronasyl residues is thought to precede cellular activation and is clearly upregulated on microglia in diseases of the CNS such as HIV and Alzheimer's disease (personal observations).

The lectins used in this study were RCA-1, GSB4 and tomato lectin. ***Ricinus communis agglutinin-1*** (RCA-1, RCA_{120}), isolated from castor bean seeds, is a glycoprotein with a molecular weight of 120KD, consisting of two subunits. RCA-1 preferentially binds to oligosaccharides ending in galactose (β-D-galactose, a common constituent), but may also interact with N-acetyl-α-D-galactosamine. Desialylation is occasionally necessary, since most glycoproteins possess terminal sialic acid residues that can block lectin binding. The

inhibitory sugar for RCA-1 is lactose or glactose. ***Griffonia (Bandeiraea) Simplicifolia Lectin-I isolectin B4*** (GSB4/BSB4) belongs to a family of glycoproteins with a MW of ~115Kd. There are two types of subunit (A and B) that combine to form tetrameric structures. The A-rich lectin preferentially agglutinates blood group A erythrocytes (specific for α-N-acetylgalactosamine residues), whereas the B-subunit preferentially agglutinates blood group B cells and is specific for α-galactose residues. GSB4 isolectin only contains the B subunits and is reported as a useful marker for endothelial cells and microglia from non-primate (mouse, rat, rabbit and goat) species. The inhibitory sugar for GSB4 is galactose. ***Lycopersicon esculentum*** (tomato lectin) is a stable glycoprotein containing 50% arabinose and glactose. It is composed of a single polypeptide of 100Kd that can form aggregates in solution. Tomato lectin preferentially binds to trimers and teramers of N-acetylglucosamine, and to a number of glycoproteins including glycophorin.

Protocol: Lectin histochemistry protocols were modified from a method previously described by Mannoji et al. (1986). Frozen sections were thawed at room temperature and air-dried for two hours, prior to immersion in a solution of methanol containing 2.5% of 30% hydrogen peroxide for one hour. Sections embedded in paraffin wax were first dewaxed in xylene, rehydrated in 100% alcohol (2 x 5 minutes) prior to immersion in the methanol/hydrogen peroxide solution. Fixed tissue sections were incubated with lysis buffer (Hanks balanced saline solution (HBSS) containing 1% of each of the following: BSA, Tween 20, [1M] $CaCl_2$, [1M] $MgCl_2$) for 90-120 minutes at room temperature, and incubated overnight at 4^0C with 1:250-500 biotinylated RCA-1, GSB-4 or 1:100-250 dilution of tomato lectin solution (Vector laboratories) made up in lysis buffer. Biotinylated lectins require calcium and magnesium ions for maximum activity, and HBSS was used as diluent, since PBS proved to have an adverse effect resulting in much weaker staining (personal observations). Following three 5-minute washes in PBS, sections were incubated with ABC-HRP (Dako, UK) for 90-120 minutes. After three final washes with PBS, lectin reactivity was visualised using peroxidase substrates: DAB or Vector VIP (Vector Laboratories, UK). Slides were dehydrated, cleared and coverslipped in the usual manner. Negative controls were included where the lectin was preincubated with its inhibitory sugar (400mM galactose/lactose/N-acetylgalactosamine/N-acetylglucosamine, 1hour at 37°C) in solution prior to incubating with sections.

Protocol 3. SILVER IMPREGNATION

Modified method after del Rio-Hortega: Vibratome-sectioned formalin-fixed tissues (20-40μm thick) were received in 25ml of deionised water to which 2 drops of ammonia were added and left for 20 minutes. Following a rapid wash with deionised water, the free-floating

sections were immersed for ten minutes at room temperature in a solution containing 5ml of 10% silver nitrate, 20ml of 5% sodium carbonate and 15ml of ammonia (sufficient to dissolve the resultant precipitate). Tissue sections were washed with deionised water, reduced in 20% non-neutralised formalin in deionised water for 1 minute, before a further wash with deionised water and toned in yellow gold choride for 1-5 minutes. They were air-dried, cleared and mounted with a glass coverslip in the usual manner. Microglia stained black against a beige-brown background. **Weil-Davenport method:** 20-40μm thick formalin-fixed cryostat or vibratomed sections were manipulated through the following solutions: (i) silver solution (18ml freshly prepared silver nitrate titrated with approximately 2ml of 30% ammonia, to dissolve the resultant preceipiate), for 20 seconds at room temperature, and following a rinse in deionised water, (ii) sections were reduced in 15% non-neutralised formalin in deionised water for approximately one minute (until sections were beige-brown in colour). Next, they were washed with deionised water, mounted onto slides, air-dried, cleared and mounted with a glass coverslip in the usual manner. Microglia stained black against a beige-brown background.

Protocol 4. IN SITU HYBRIDISATION

1. Selection of RNA primers and oligonucleotide probes

Complete sequences encoding mRNA for GFAP (control), and the chemokines MCP-1 and MIP-1α were accessed from the NIH GenBank and SwissProt databases:-

GFAP GenBank ID (Accession numbers: JO4569 [GDB:G00-118-799];
Protein Id [AAA52528.1]; PID: g183075; GI: 183075.

MCP-1 GenBank ID (Accession): M31626; M24545; (NM 002982); NID: g4506840;
Protein Id: NP 002973.1 (P13500- Swiss-Prot); PID: g4506841 (g307163);
GI: 4506841 (307163).

MIP-1α GenBank ID (Accession): M23452 (NM 002983); NID: g4506843; GI: 4506843;
NP 002974.1; PID: P10147 (Swiss-Prot)

Possible sequences that could act as primers, or had been previously used as primers in the literature, were identified within the encoding region. A 30 oligomer was selected within this encoding region encompassed by the primer sequences:-

Note: sequences are presented in 5'-3' direction.

	GFAP	Position in Database Entry
Forward Primer	ACT CAA TGC TGG CTT CAA GGA	74-194
Reverse Primer	CCC GCA ACG CGG AGC TGC TCC	809-829
Selected sequence	GCA GAT GAA GCC ACC CTG GCC CGT CTG GAT	529-558
Oligonucleotide Probe	ATC CAG ACG GGC CAG GGT GGC TTC ATC TGC	
Specification of Probe	Tm 68.3°C; MW 9195; OD/ml (@260nm) 2.80; 92.40µg/ml; 8.9pmol/µl	

	MCP-1	Position in Database Entry
Forward Primer	ATG AAA GTC TCT GCC GCC CTT	70-90
Reverse Primer	ACC CAA ACT CCG AAG ACT TGA	349-369
Selected sequence	GTG CAG AGG CTC GCG AGC TAT AGA AGA ATC	202-231
Oligonucleotide Probe	GAT TCT TCT ATA GCT CGC GAG CCT CTG CAC	
Specification of Probe	Tm 64.2°C; MW 9080; OD/ml (@260nm) 2.70; 89.10µg/ml; 9.0pmol/µl	

	MIP-1α	Position in Database Entry
Forward Primer	ATG CAG GTC TCC ACT GCT GCC	84-104
Reverse Primer	GAC CTG GAG CTG AGT GCC TGA	342-362
Selected sequence	CGG CAG ATT CCA CAG AAT TTC ATA GCT GAC	201-230
Oligonucleotide Probe	GTC AGC TAT GAA ATT CTG TGG AAT CTG CCG	
Specification of Probe	Tm 61.5°C; MW 9208; OD/ml (@260nm) 3.00; 99.00µg/ml; 9.2pmol/µl	

Oligonucleotide probes have been used to detect genes, short sequences and RNA. The above probes were synthesised and purchased from Perkin-Elmer Applied Biosystems, UK, supplied ready to use in 20% acetonitrile/water. The melting temperature (Tm) of the probe (the temperature at which one half of the duplex molecules become dissociated into single strands) can be estimated from the constituent bases (A+T: 2 OH bonds; G+C: 3 OH bonds). Tm is known to be influenced by the salt concentration, the percentage of formamide (a destabilising molecule that disrupts hydrogen bonds) in the hybridisation solution, the probe length and GC content of the probe. As a simple rule, Tm (^{0}C) = 2 x (number of AT base pairs) + 4 x (number of GC base pairs). Low salt and high formamide concentrations assist further in producing high stringency (refer to procedure below). 30-base oligonucleotide probes were selected with high Tm. Formamide used in the protocol decreased the melting temperature by breaking OH bonds, reducing Tm to ~50^{0}C.

A Basic Local Alignment Search Tool (BLAST) engine was used to explore similarities between the selected mRNA sense sequences (against which the oligonucleotide probes were developed) and all available formats on the databases. This search was accessed via the National Center for Biotechnology and Information web site (NCBI, NIH, Betheseda, USA: http://www.ncbi.nlm.nih.gov/UniGene/). The specificity of the probes for their respective proteins was assessed in this manner. The 30-base mRNA sequence selected for GFAP [request ID: 941101040-21140-27537] was 100% homologous with records of human GFAP sequences. The MCP-1 mRNA sequence [request ID: 941190920-20286-11027] was 100% homologous with recorded MCP-1 sequences. The MIP-1α mRNA sequence [request ID: 941190665-18743-13384] showed 95-100% homology with human MIP-1α sequences.

IN SITU HYBRIDISATION: Protocol for frozen sections **[light microscopy]**

In situ hybridisation was performed on frozen sections of foetal and adult human CNS, using the non-radioactive *in situ* hybridisation oligo colour kit from Amersham International Plc, UK (catalogue #RPN 3400). This method detects fluoresceinated oligonucleotide probes using a secondary antibody reagent targeted to fluorescein, conjugated with alkaline phosphatase, and binding subsequently detected using nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) as substrate, according to standardised protocols (Wisden and Morris, 1994). All solutions were prepared in RNAse-free autoclaved water (18.2MΩ, endotoxin-free, ultrapure filtered, Millipore, UK). The kit comprised the following: (i) 50µl fluorescein-11-dUTP, (ii) 200 units of terminal transferase in 100µl of buffer, pH7.0, (iii) 100µl of 10x concentrated buffer containing sodium cacodylate, pH7.2, (iv) 1ml of sterile deionised water, (v) control unlabelled probe: 300ng/30µl M13 forward sequencing primer, (vi) anti-fluorescein alkaline phosphatase (AP) conjugate: 40µl of a 1000x concentrated stock of sheep anti-fluorescein antibody conjugated to alkaline phosphatase, (vii) 5ml of hybridisation buffer (1.2ml sterile water; 1ml of 20x SSC (see below), 0.5g dextran sulphate (a polymer which accelerates the rate of hybridisation reaction by up to 3 times, by forming a matrix in the hybridisation mixture which concentrates the probe without affecting the stringency: an estimate of the approximate percentage of nucleotides that are correctly matched between probe and target sequences), 100µl of 50x Denhardt's solution (2g Ficoll, 2g polyvinyl pyrollidone, 2g bovine serum albumin, fraction V dissolved in 100ml sterile water and aliquoted), 2.5ml deionised formamide (regulates stringency and lowers Tm), 400ml of a stock [10ng/ml] solution of denatured/sonicated salmon sperm DNA (unlabelled blocking DNA to block probe hybridisation to non-specific sites), (viii) 10g of blocking agent, (ix) 500µl of BCIP solution in dimethylformamide, (x) 500ml of NBT solution in 70% dimethylformamide.

Preparation of fluorescein-labelled probe: The following protocol was employed for single-stranded oligonucleotides bearing 3'-OH group. Original stock concentrations of the probes were: [9.0 pmoles/µl] MCP-1, [9.2pmoles/µl] MIP-1α, [8.9pmoles/µl] GFAP. The fluorescein-labelling reaction was catalysed by terminal deoxinucleotidyl transferase (TdT), which introduces a tail of fluorescein-dUTP onto the 3' end of an oligonucleotide. Components of the kit were first thawed on ice. The following were added to a 1.5ml tube: 100 pmoles of the oligonucleotide probe, 10µl of fluorescein-11-dUTP, 16µl of the cacodylate buffer, 16µl of terminal transferase, and deionised water supplied to obtain a final total volume of 160µl. The solution was mixed gently by pipetting, and the reaction mixture incubated at 37^0C for 60-90 minutes. Labelled probes were stored on ice or at -20^0C for long-term storage (stable up to six months). The optimised reaction protocol was considered to yield a tail length sufficient to ensure high sensitivity in hybridisation without compromising the stringency of the specific probe sequence. An incubation time of 1 hour was typically sufficient to label 25-250pmoles of probe, and there was no need to purify unincorporated fluorescein-dUTP from labelled oligonucleotide.

Procedure: Frozen sections previously cut ~20µm thickness onto extra-clean RNAse-free positively-charged slides (VWR international Ltd.), were thawed to room temperature, and fixed in freshly prepared 4% paraformaldehyde solution in TBS. Sections were next rinsed in TBS and treated as follows. **Pretreatment:** They were rinsed in ultrapure water, placed in TBS for 2 minutes, and permeabilised with 0.1-0.5mg/ml of Proteinase K (made up in Tris-EDTA buffer, comprising 10mM Tris-HCl, 1mM EDTA, pH 8.0, prewarmed at 37^0C for 10 minutes, 100-200µl per slide). Sections were incubated in this solution for 20-30 minutes at 37^0C. Digestion was terminated by rinsing sections twice with ultrapure water and washing with a solution of glycine (2mg/ml) in TBS for 5 minutes. Next, sections were washed in cold 20% acetic acid (stored in the refrigerator) for approximately 15-20 seconds to remove endogenous alkaline phosphatase, and rinsed twice (5 minutes per wash) with TBS. **Hybridisation:** Sections were allowed to air-dry for twenty minutes before proceeding with hybridisation. Briefly, the hybridisation buffer was prewarmed at 37^0C for 5 minutes, and carefully mixed 1:1 with deionised formamide. The labelled probe solution [50ng/ml] was added to 1ml of hybridisation buffer, and 20-40µl of the mixture placed onto tissue sections. A parafilm cover was applied to ensure even coverage of the section by the hybridisation mixture, and slides were placed on a hotplate at 90^0C for ten minutes, following which they were incubated at 37^0C for 16-24 hours. The parafilm was removed with care and sections placed in TBS containing 0.1% Triton X-100 twice, three minutes per wash. **Stringency:** Sections were next rinsed twice (5 minutes per wash) in 1 x SSC (diluted from a 20x stock comprising 3M NaCl, 0.3M trisodium citrate, pH7.0 with HCl, which was filtered and

autoclaved), and 0.1% (w/v) SDS (lauryl sulphate sodium salt/sodium dodecyl sulphate, molecular biology grade, Sigma, UK). Sections were washed thoroughly with TBS to remove the SDS. **Detection:** Sections were then incubated with blocking solution (1:5 normal rabbit serum (Dako, UK) made up in TBS containing 3% BSA and 0.1% Triton X-100) for 30 mins. The blocking solution was discarded, sections rinsed briefly with TBS, and incubated with rabbit F(ab) anti FITC conjugated to alkaline phosphatase (Dako, UK; diluted 1:100 in TBS containing 3% BSA, 0.1% Triton X-100) for one hour. Sections were then washed twice in TBS (3 minutes per wash), and rinsed for 5 minutes in optimised detection buffer (100 mM Tris-HCl; 50 mM $MgCl_2$; 100 mM NaCl; pH 9.0). Alkaline phosphatase activity was demonstrated by incubating sections overnight (in the dark, at room temperature) in NBT/BCIP substrate (45µl NBT, 35µl BCIP added to 10ml detection buffer, containing 40% dimethylformamide solution and 1µl/ml levamisole which inhibit endogenous alkaline phosphatase activity). Sections were next washed in running tap water, air-dried and mounted in permount from microclear (Raymond Lamb, UK) or with PBS/glycerol mountant. **Controls:** Negative controls were included where (i) sections were incubated with hybridisation buffer lacking probe, (ii) the mRNA binding site was hybridised with an *unlabelled* probe, or (iii) the M13 forward sequencing primer (30µl of a 10ng/ml stock) replaced the oligonucleotide probe in the hybridisation buffer. An oligonucleotide probe to GFAP mRNA sequence was used as a positive control for the procedure.

Protocol 5. IN SITU CELL DEATH DETECTION

TdT-mediated dUTP end labelling: The modes of cell death: apoptosis or necrosis, can be distinguished according to morphological, biochemical and molecular criteria. Programmed cell death (apoptosis) represents an apparently natural mechanism in tissue turnover and during development. Apoptosis occurs without accompanying inflammatory consequences, which are usually associated with necrotic events. Instead, cells undergoing apoptosis appear to disassemble nuclear and cytoplasmic components in an organised manner. Endonucleolysis, which results in the unravelling and cleavage of nuclear DNA into oligonucleosome-sized fragments, is considered as a central event in programmed cell death or apoptosis. This process can be detected as a 'DNA ladder' on agarose gels during electrophoresis. The *in situ* labelling of aooptosis-induced breaks in the DNA strand is useful for identifying apoptosis in individual cells and to determine their histological localisation. DNA polymerase and terminal deoxyribonucleotide transferase (TdT) have been widely used for the incorporation of labelled nucleotides to DNA strand breaks *in situ*. This 'tailing' reaction using TdT (also described as ISEL: *in situ* end labelling; or TUNEL: TdT-mediated dUTP nick end labelling) has been used

here, as outlined in the instruction manual of the *In Situ* Cell Death Detection Peorxidase Kit (Roche Diagnostics, GmbH, Cat No. 1 684 817).

The test principle is relatively simple: cleavage of genomic DNA during apoptosis may yield double-stranded, low molecular weight DNA fragments (mono- and oligo- nucleosomes) as well as single-strand breaks ('nicks') in high molecular weight DNA. These breaks in DNA strands can be identified by labelling free 3'-OH termini with modified nucleotides in an enzymatic reaction. TdT catalyses polymerisation of nucleotides to free 3'-OH termini of DNA in a template-independent manner, and labels DNA strand breaks. The incorporated fluorescein is detected by an antibody Fab fragment (from sheep) raised against fluorescein, and conjugated with horseradish peroxidase (HRP).

The reagents consisten of the following: (i) 250µl of enzyme solution (TdT from calf thymus in 10x concentrate storage buffer), (ii) 2,750µl of label solution (fluorescein-labeled nucleotide mixture in reaction buffer, (iii) peroxidase-conjugated sheep anti-fluorescein antibody, (iv) pre-treatment: 0.5% pepsin made up in PBS. Negative controls included incubation with label solution alone. Positive controls included incubating tissue sections with 100µg/ml deoxyribonuclease-1 (from bovine pancreas) (Sigma, UK), for 30 minutes at room temperature, to induce breaks in the DNA strand. Positive control tissue from cases with motor neuron disease and multiple sclerosis were also used, which possessed a high index of apoptosis.

Procedure: Frozen sections (20µm thick) were thawed at room temperature and air-dried, prior to immersion in a solution of methanol containing 2.5% of 30% hydrogen peroxide for one hour. Sections emebedded in paraffin wax (10-20µm thick) were first dewaxed in xylene and placed in 100% alcohol (2 x 5 minutes), prior to immersion in the methanol/hydrogen peroxide solution. Following this, tissue sections were rinsed in deionised water for 5 minutes, and pretreated in a humidity chamber with 0.5% pepsin in PBS for one hour at 37^0C. Non-specific binding was blocked by incubating sections with 1% bovine serum albumin made up in HBSS, and 1:10 dilution of normal sheep serum (Vector laboratories, UK). Positive control slides were incubated at this stage with 100µg/ml DNAse-1 (Sigma, UK) for 30 minutes at room temperature. Negative control and test sections were incubated for a further 30 minutes with the blocking solution. Next, the sections were rinsed twice in PBS (5 minutes per wash) during which the TUNEL reaction mixture was prepared on ice by adding 50µl of the enzyme to 500µl of the label solution. 50µl of the TUNEL mixture was applied to each section, and these were overlaid with parafilm to ensure even coverage, and incubated for 1-2 hours at 37^0C in a humidity chamber. Negative control slides were incubated with 50µl of label

solution alone. The parafilm was gently removed from slides and sections washed three times (5 minutes per wash) in PBS, prior to incubation with the peroxidase-conjugated sheep anti-fluorescein antibody (50µl per section, 1:5 dilution) for one hour at 37^{0}C in a humidity chamber. Next, tissue sections were rinsed three times with PBS (3 x 5 minute washes) and developed with DAB solution, dehydrated, cleared and mounted with a glass coverslip as per immunohistochemical protocols.

Protocol 6. THE STAMPER-WOODRUFF ASSAY

The method devised by Stamper and Woodruff in 1976, modified after Vora et al. (1995), was adapted in this investigation to assess the adhesion of monocytes to sections of human foetal and adult CNS. In their original studies, Stamper and Woodruff (1977) showed the selective binding of leukocytes to high endothelial venules in frozen sections of rodent lymphoid tissues. This technique was later applied for studying leukocyte-endothelial interactions in murine choroid plexus (Steffen et al. 1996), in encephalitic brain sections from rhesus monkeys (Sasseville et al. 1994), in human lymphoid tissue (Pals et al. 1986), human skin in psoriasis (Sackstein et al. 1988) and human synovium in rheumatoid arthritis (Jalkanen et al. 1986). Vora and colleagues (1995) further adapted the method to study the interaction of lymphocytes with cerebral vessels, when overlaid onto cryostat sections of the human brain.

Cell Culture: The human leukaemic monocytic cell line THP-1 (Tsuchiya et al. 1980), was obtained from the European Collection of Cell Cultures, Centre for Applied Microbiology and Research, Porton Down, Salisbury, Wiltshire, UK. This cell line was derived from the blood of a boy with acute monocytic leukaemia, and following characterisation demonstrated Fc and C3bi receptors, but lacked surface or cytoplasmic immunoglobulins. The HLA haplotype was HLA-A2, A9, B5, DRW1 and DRW2. The monocytic nature of this cell line was further supported by the ablity of THP-1 cells to phagocytose latex particles and sensitised erythrocytes, their production of lysozyme and alpha-naphthyl butyrate activities, and by their abilities to sensitise T-lymphocytes. Importantly, these cells were found to maintain their monocytic characteristics for over 14 months when maintained in culture (Tsuchiya et al. 1980). THP-1 cells were cultured in 75cm^3 flasks with RPMI-1640 medium (Gibco, UK) supplemented with 5% foetal calf serum, 100 units/ml penicillin, 100 µg/ml streptomycin, 2mM glutamine, and 2 x 10^{-5}M β-mercaptoethanol, in an incubator at 37^{0}C, with 5% CO_2. THP-1 cells were grown in suspension to a density of 1 x 10^6 cells/ml prior to use.

In situ adherence assay: Cell suspensions were harvested by centrifugation at 4^{0}C (12,500 rpm, for 5 minutes), washed twice with with modified HBSS (without calcium or magnesium),

before being resuspended in HBSS at a density of 2 x 10^6 cells/ml for the Stamper-Woodruff assay. Fresh-frozen cryostat sections were allowed to thaw at room temperature for 20 minutes, before being overlaid with 200µl of cell suspension (4 x 10^5 THP-1 cells), and incubated for one hour at 37^{0}C. Through varying the cell densities (1 x 10^6 down to 1 x 10^4 cells per section), temperature (4^{0}C in the refrigerator, 19-21^{0}C at room temperature, and in the incubator at 37^{0}C) and duration of incubation (10-90 minutes), the conditions selected were optimised to result in reproducible binding patterns of THP-1 cells to the tissue sections. Following the incubation, sections were washed carefully with HBSS (with calcium and magnesium) to remove non-adherent cells, and fixed either with 1% paraformaldehyde or pure methanol solution containing 2.5% of a 30% hydrogen peroxide solution for 90 minutes, and processed for light microscopic evaluation of cells bound to the tissue. Sections were checked under the light microscope at each of the above stages, in order to determine the extent of possible detachment of bound cells. There was no appreciable loss of bound THP-1 cells when following this procedure. In order to determine the specificity of binding, control tissue sections were incubated with the following prior to being overlaid with THP-1 cells: (i) normal HBSS, (ii) modified HBSS (calcium and magnesium-free) with 1-5mM EDTA (a calcium chelating agent which inhibits integrin adhesion), or (iii) a cocktail of inhibitory synthetic proteins targeting integrins (200µg/ml): RGD (targeting collagen/laminin, α3β1 integrin), EILDV (targeting fibronectin, α4β1 integrin), QIDSP (targeting fibronectin, α5β1 integrin). Sections of the brain from normal adults and multiple sclerosis cases were used as controls.

Lectin Histochemistry: Fixed tissue sections were incubated with lysis buffer (HBSS containing 1% of each of the following: BSA, Tween 20, [1M] $CaCl_2$, [1M] $MgCl_2$) for 90 minutes at room temperature, and incubated with 1:500 biotinylated RCA-1 solution made up in lysis buffer (Vector Laboratory, UK) for 4 hours. Following three 5-minute washes in HBSS, sections were incubated with ABC-HRP (Dako, UK) for a further 90 minutes. After three washes with PBS, RCA-1 reactivity was visualised using the peroxidase substrate: Vector VIP (Vector Laboratories, UK). Slides were dehydrated, cleared and coverslipped in the usual manner. Negative controls were included where the lectin was preincubated with its inhibitory sugar (400mM galactose, 1hour at 37^{0}C) in solution prior to pipetting onto sections.

Immunohistochemistry: Single and dual-label immunohistochemistry was performed to identify mononuclear phagocytes and adhesion molecules as previously outlined. For dual-label studies, the standard protocol was adhered to, with the following exception: sections were first incubated with antibodies to CD45, CD64, CD68 or with a cocktail of these monoclonal antibodies (CD45:CD64:CD68, all at 1:20) for 4 hours at room temperature, and

developed using DAB as chromogen to give a brown reaction product. Subsequently, sections were incubated with antibodies to detect adhesion molecules (**Table 6**) for 4 hours at room temperature, and developed using Vector VIP (Vector Laboratories, UK) to give a violet reaction product. If required, sections were lightly counterstained with methyl green.

RESULTS

I. Distribution, phenotype and morphology of microglia in the human brain during the second trimester

The structural integrity of tissue sections was well-preserved despite the rigorous procedures that were followed. Anatomical localisation and terminology were determined from a standard reference atlas of the human foetal brain (Feess-Higgins and Larroche, 1979), and through the assistance of a resident paediatric neuropathologist (Dr. Andrew Dean), within the Department of Neuropathology, Institute of Psychiatry. During development, the cerebral cortex (telencephalon) is characterised by five histological layers that develop by the third month of foetal life (8-10 weeks) see **Figure 1** (please refer also to **Figure 5, Figure 6** and **Figure 21**). The germinal layer, lining the ventricles is composed of a densely nucleated ventricular zone (VZ) and overlying subventricular zones (SVZ), and represents the major site for gliogenesis and neurogenesis. Overlying these is the intermediate zone (IZ), a transitory structure, which in the future forms the white matter, through which glial and neuronal progenitors migrate to reach the cortical plate (CP). The marginal layer (ML), containing the Cajal-Retzius cells which direct the laminar distribution of neurons in the CP, is placed between the CP and the pial surface. The subplate (SP) is another transient structure located immediately below the CP, which harbours thalamo-cortical fibers, as these develop and make their appropriate connections within the laminae of the CP.

Operational criteria used to define populations of microglia in the developing brain

According to the observations discussed below, several characteristic morphologies and phenotypes of microglia could be defined within the developing brain. (i) $CD68^{+}/RCA\text{-}1^{+/-}$ and $CD68^{++}/RCA\text{-}1^{+}$ cells with morphologies more typical of macrophages (large, rounded or amoeboid vesicle-laden cells with an uneven or 'ruffled' surface membrane) were referred to as 'amoeboid' microglia. These were typically found in the meningeal and marginal layers and confined in limited numbers immediately subjacent to the cortical plate of the telencephalon, within white matter tracts and close to cerebral blood vessels deeper areas of the brain. (ii) A population of $RCA\text{-}1^{+}/CD68^{-}$ cells smaller in size and of rounded (undifferentiated) appearance which mainly resided in the marginal layer, the cortical plate and subplate at the outset of the second trimester were referred to as 'microglial progenitors' or 'precursor cells'. These cells were freely lying in the marginal layer, closely associated with the ablumenal wall of cortical blood vessels, and only occasionally detected in the corpus callosum and within deeper regions of the developing brain, again in association with small blood vessels.

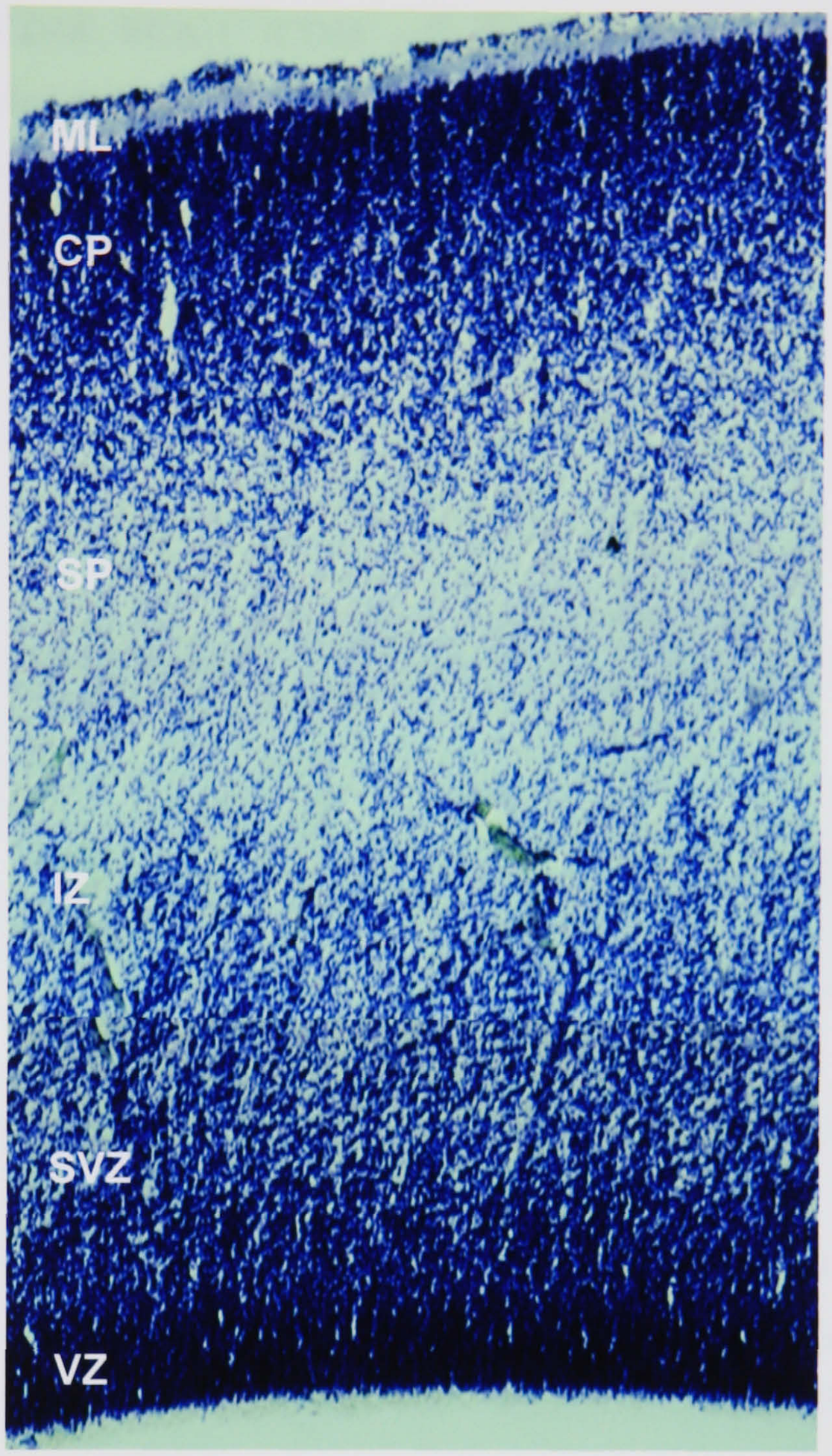
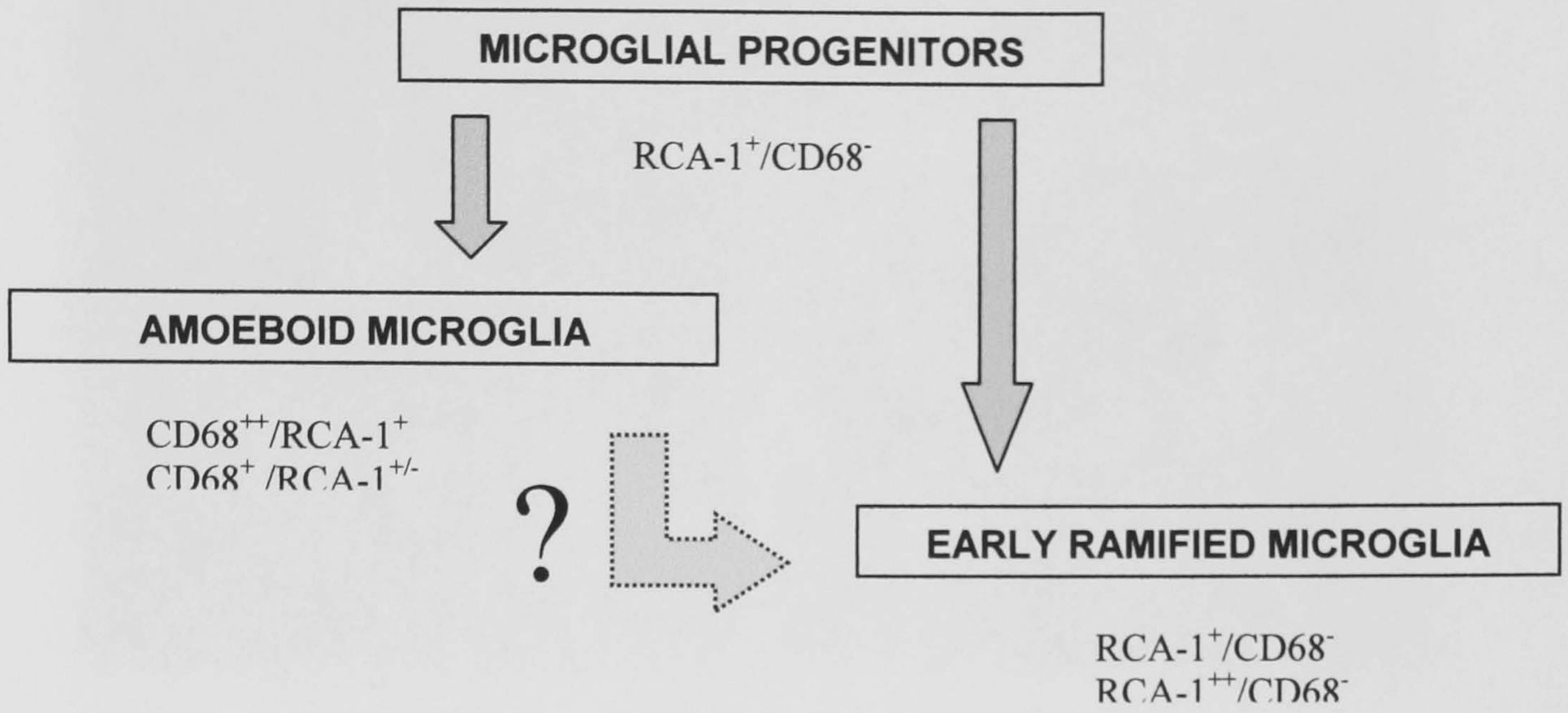


FIGURE 1

A Nissl-stained section taken through the telencephalon at 13GW to show the five characteristic histological layers

The germinal layer, lining the ventricles is composed of a densely nucleated ventricular zone (**VZ**) and overlying subventricular zones (**SVZ**), and represents the major site for gliogenesis and neurogenesis. Overlying these is the intermediate zone (**IZ**), a transitory structure, which in the future forms the white matter, through which glial and neuronal progenitors migrate to reach the cortical plate (**CP**). The marginal layer (ML), containing the Cajal-Retzius cells which direct the laminar distribution of neurons in the CP, is interposed between the CP and the pial surface. The subplate (**SP**) is another transient structure located immediately below the CP, which harbours thalamo-cortical fibers, as these develop and make their appropriate connections within the laminae of the CP.

(iii) The terms 'transforming', 'transitional' and 'early ramified' microglia refer to RCA-1^{+}/$CD68^{+}$, RCA-1^{+}/$CD68^{-}$, RCA-1^{++}/$CD68^{-}$ cells that had begun to differentiate (ramify) and more closely resembled the adult ramified microglia in morphology (see also **Figure 21**). The term 'mononuclear phagocyte' encompasses all these different characteristic forms of developing microglia.



The precise relationship between amoeboid cells and early ramified microglia is difficult to determine from the studies *in situ*. As discussed below, the amoeboid 'state' appears to be bypassed at least during the first phase of colonisation of the developing telencephalon that takes place before 16GW, where by far the majority of microglial progenitor cells begin to differentiate morphologically and ramify without apparently passing through an amoeboid stage.

12-14 gestational weeks

Telencephalon

Immunohistochemsitry with the macrophage marker CD68 revealed weak-to-moderate expression on an early population of mononuclear phagocytes residing within the marginal layer and subplate of the telencephalon (**Figure 2**). Some cells were interspersed throughout the cortical plate, particularly within the lower aspect of this layer. Immunoreactivity with CD68 was more intense on round cells located within the upper marginal layer, underlying the meninges at 13GW. Corresponding higher power photographs of CD68 positive cells located within the marginal/pial layers and the subplate are presented in **Figure 2** and **Figure 3** respectively. The majority of CD68 positive cells located within the upper aspect of the marginal layer (underlying the pial surface) were of round shape, up to 25-30μm in diameter with an uneven surface and morphology typically resembling macrophages (**Figure 2B,C**).

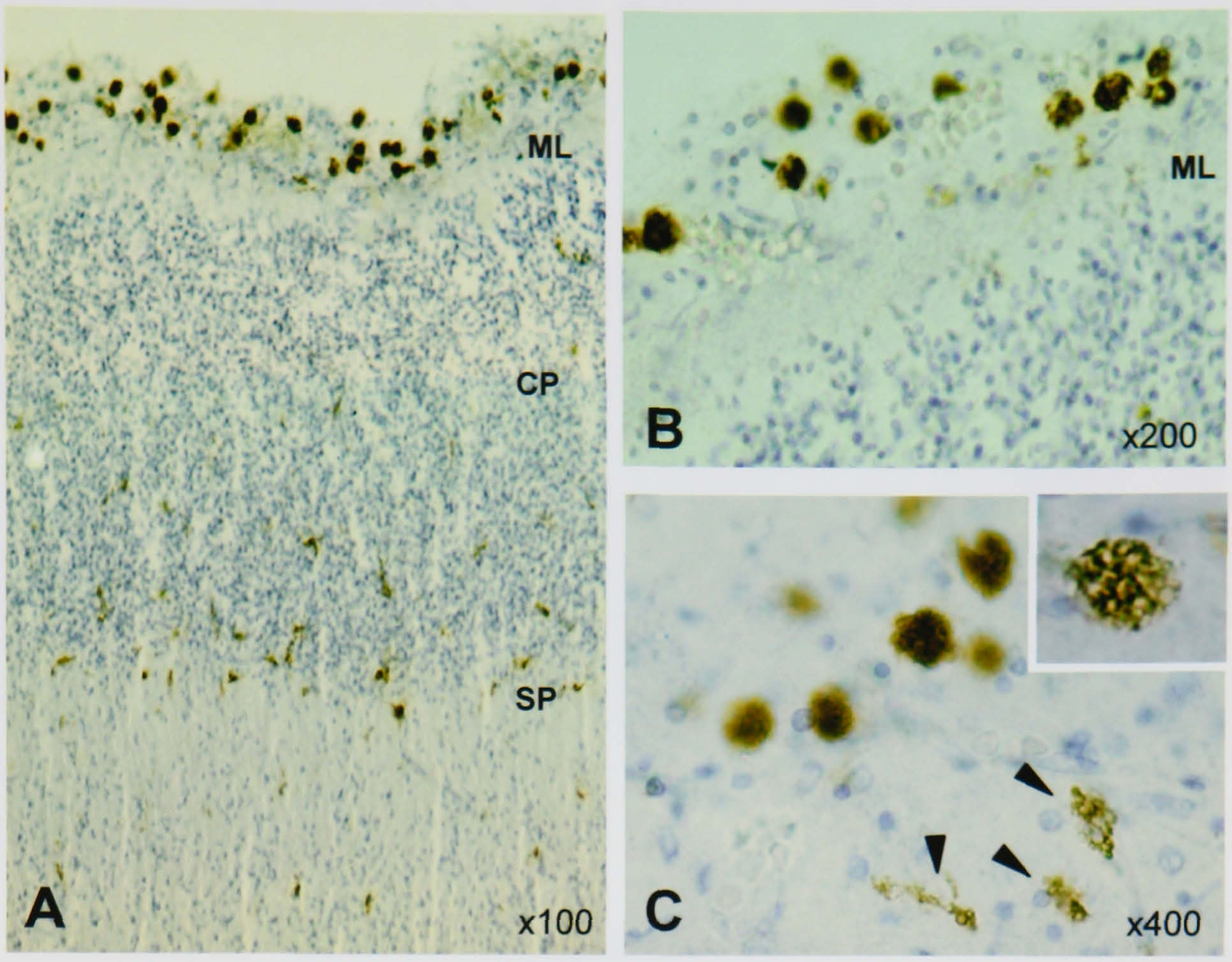


FIGURE 2

CD68 positive cells in the human foetal telencephalon at 13GW

CD68-immunoreactive cells are located within the marginal layer (ML) below the pial layer of the meninges, interspersed throughout the cortical plate (CP) with particular preference for lower cortical layers, and within the subplate (SP) of the telencephalon (**A**). CD68 positive cells are conspicuously absent within other layers of the telencephalon. (**B,C**) Higher power photos of CD68-immunoreactive cells located within the ML. The majority of these rounded mononuclear phagocytes resemble monocytes/macrophages in their morphology (**C, inset**). They are frequently detected around small meningeal vessels, particularly overlying the marginal layer. Occasionally, more differentiated, CD68-immunoreactive cells of smaller amoeboid morphology can be seen within the marginal layer itself (**C, arrows**) and within the upper layers of the cortical plate. Nuclei counterstained with haematoxylin or methyl green.

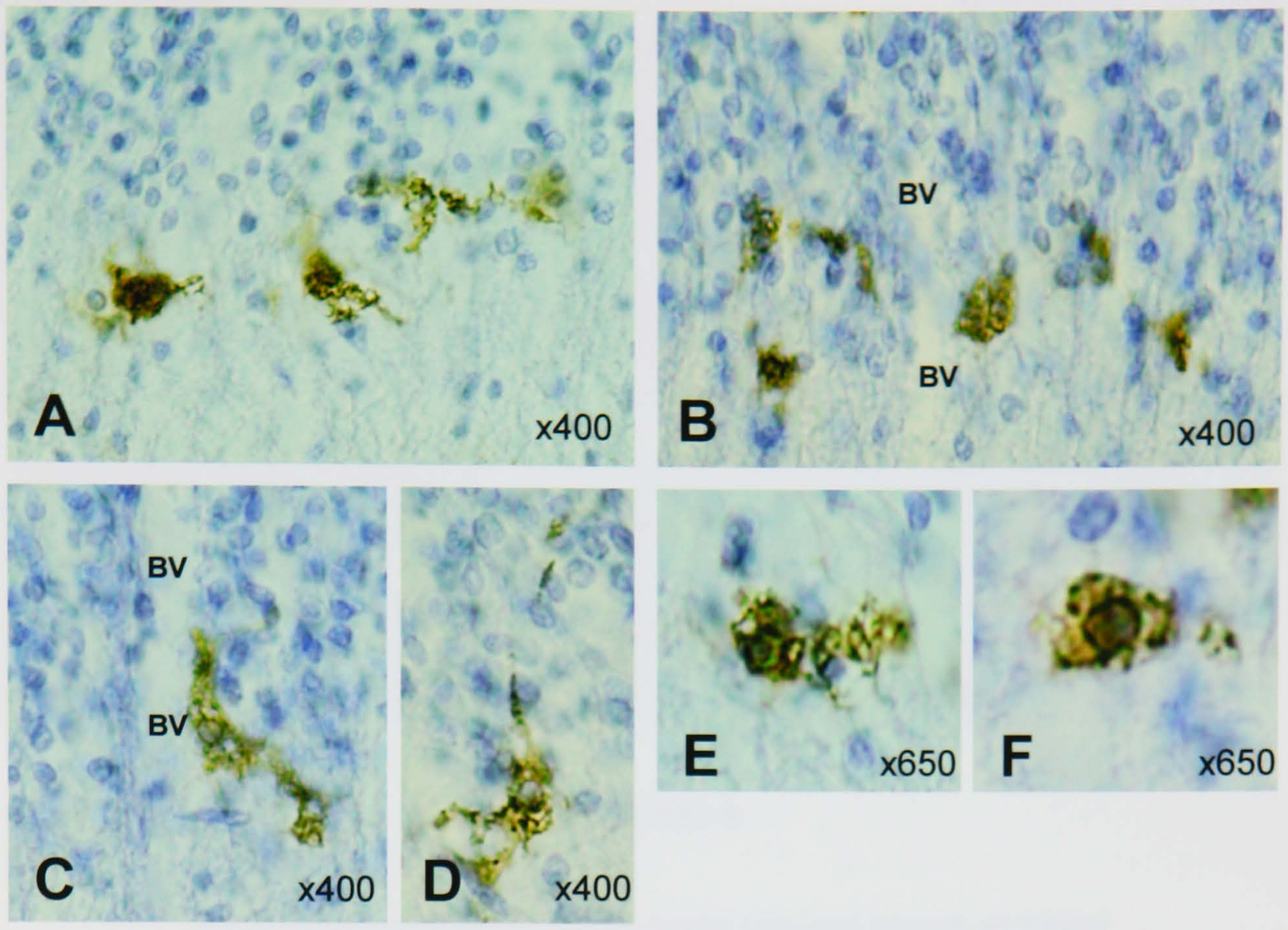


FIGURE 3

CD68 positive cells in the subplate of human foetal telencephalon at 13GW

Higher power photos of CD68-immunoreactive cells confined within the subplate. These cells are clearly amoeboid in morphology (**A-F**), associate with radial blood vessels (BV) passing through the cortical plate and subplate (**B,C**), and occasionally appear more ramified, extending processes several micrometers away from the cell body (**D**). Expression of CD68 is both at the cell surface and of a vesicular pattern within the cytoplasm (**E-F**), in keeping with previous descriptions of macrophages. Nuclei counterstained with haematoxylin.

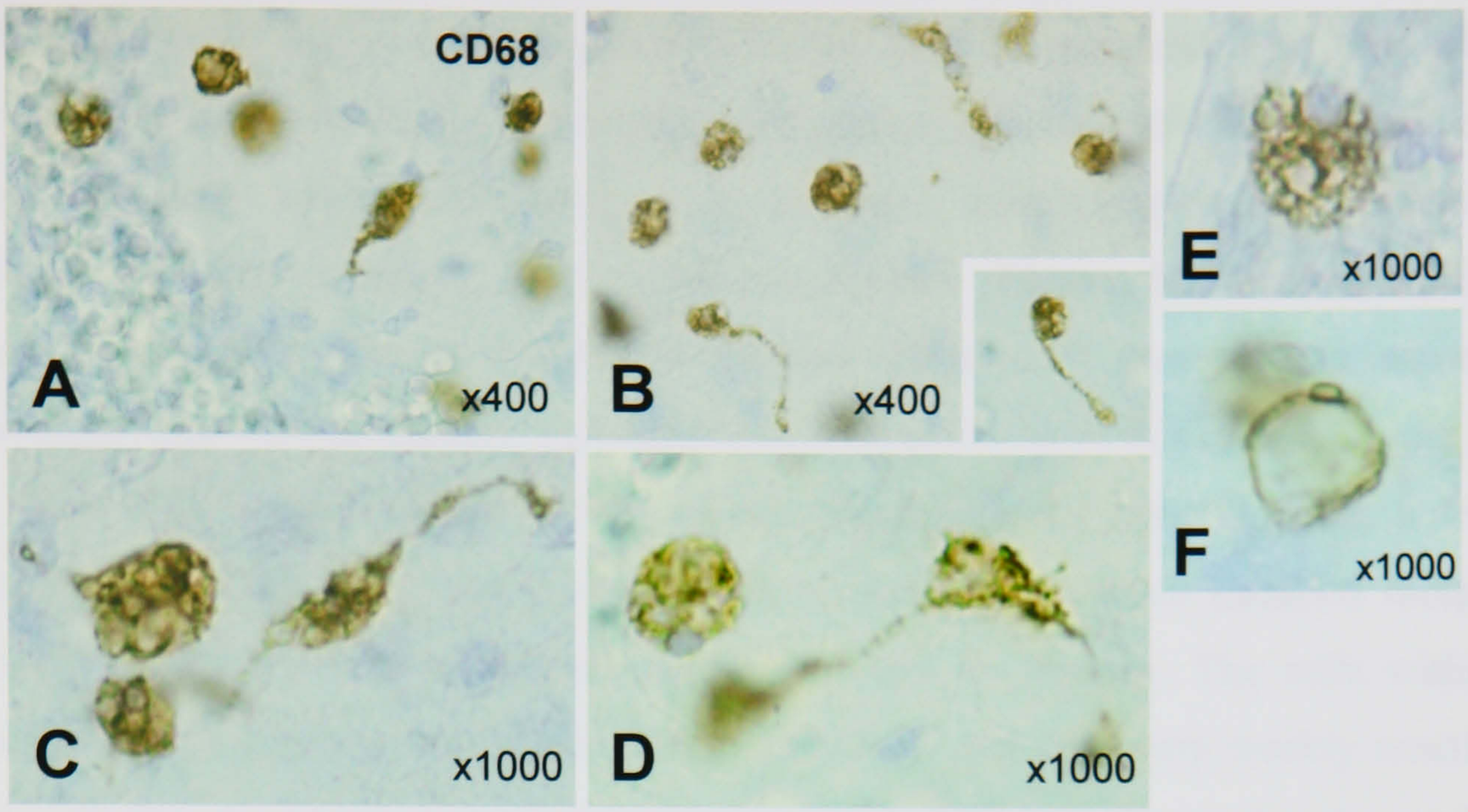


FIGURE 4

CD68 positive cells in human foetal choroid plexus at 13GW

CD68 immunoreactive cells located within the choroid plexus demonstrate heterogeneous morphologies, which include round, unipolar and bipolar varieties (**A-D**). Cells resembling macrophages (**E**) with granular cytoplasm and rough membrane are common within the choroid at this gestational age. Occasionally, cells are encountered that express CD68 only at their periphery (**F**), and usually possess displaced nuclei. These morphological characteristics are more typical of mononuclear phagocytes undergoing cell death, a phenomenon which will be further elaborated in Chapter IV.

Many of these were located around (but not usually within) delicate, small-diameter and thin-walled meningeal vessels. With progression further into the marginal layer, and within the upper limits of the cortical plate, a more differentiated morphology of CD68 positive cells was evident, and these smaller cells adopted an amoeboid morphology more characteristic of amoeboid microglia according to previous descriptions (**Figure 2C, arrowheads**). CD68 immunoreactive cells located within the subplate (**Figure 3**) were clearly amoeboid in morphology, and showed surface and cytoplasmic reactivity with this marker. Some of these cells were also located around blood vessels penetrating the cortical plate. a few cells appeared in pairs and very rarely cells could be seen with a more ramified morphology, extending two or more processes several micrometers in distance. The cells within these regions were not immunoreactive for CD11b or CD64. and only very weakly reactive with CD45 (not shown). CD68-immunoreactive cells were distinctly absent elsewhere within the developing telencephalon. However, positivity for this marker was clearly demonstrated on mononuclear phagocytes within the choroid plexus (**Figure 4**). Within the choroid, a morphologically heterogeneous population of cells coexisted, with round, unipolar, bipolar and amoeboid shapes. Cells with morphological characteristics typical of macrophages (rough membrane, cytoplasmic granules), were frequent. Occasionally a cell was encountered that expressed CD68 only at the periphery and whose nucleus was displaced to one side of the membrane. These were more typical signs associated with a form of cell death in mononuclear phagocytes, a phenomemon which will be further elaborated upon in Chapters IV and V.

Lectin histochemistry with RCA-1 highlighted a population of progenitor cells within the marginal layer, cortical plate and subplate of the telencephalon, which could be distinguished morphologically from the CD68-immunoreactive cells already described (**Figures 5-8**). **Figure 5** and **Figure 6** show lower power composite photomicrographs of the entire expanse of the telencephalon between 13-14 GW, histochemically reacted with RCA-1. From these figures, it is clear that the accumulation of cells within the lower cortical layer and subplate (indicated by arrowheads) represent the first microglial colony within the telencephalon. Microglia, which are at various stages of differentiation in the subplate, could be clearly and readily discriminated from blood vessels which were also stained with the lectin. At this stage, the ventricular, subventricular and intermediate zones are largely devoid of microglial progenitors. There was a greater density of transforming RCA-1 positive cells within the subplate and upper aspect of the intermediate zone by 14GW (**Figure 6**). RCA-1 positive cells in the marginal layer and cortical plate were small in diameter, and round or ovoid within the marginal layer. occasionally occurring as paired cells, and associated with the parenchymal aspect of radial blood vessels that penetrated the cortical plate (**Figure 7**).

TELENCEPHALON

RCA-1

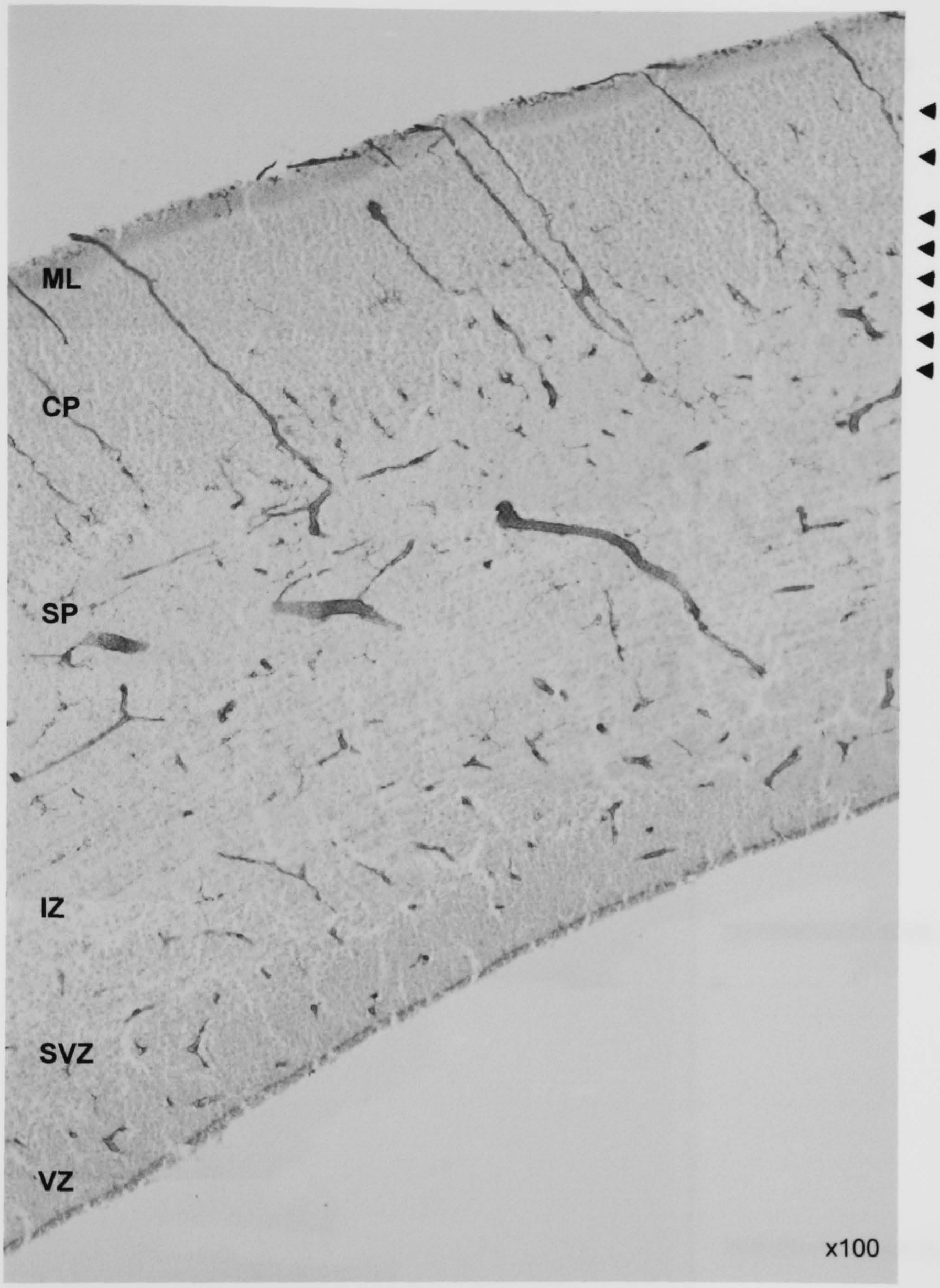


FIGURE 5

RCA-1 histochemistry within the human foetal telencephalon 13-14GW

Lectin histochemistry with RCA-1 identifies blood vessels and microglial progenitors within the telencephalon between 13-14 GW (see also figure 6). Microglial progenitors and early transforming ramified microglia can be found in the lower cortical plate and subplate (arrowheads, see Figures 8-10), whereas the intermediate zone, subventricular and ventricular areas are largely devoid of microglia or their progenitor cells during this period. (ML) marginal layer, (**CP**) cortical plate, (**SP**) subplate, (**IZ**) intermediate zone, (**SVZ**) subventricular zone, (**VZ**) ventricular zone.

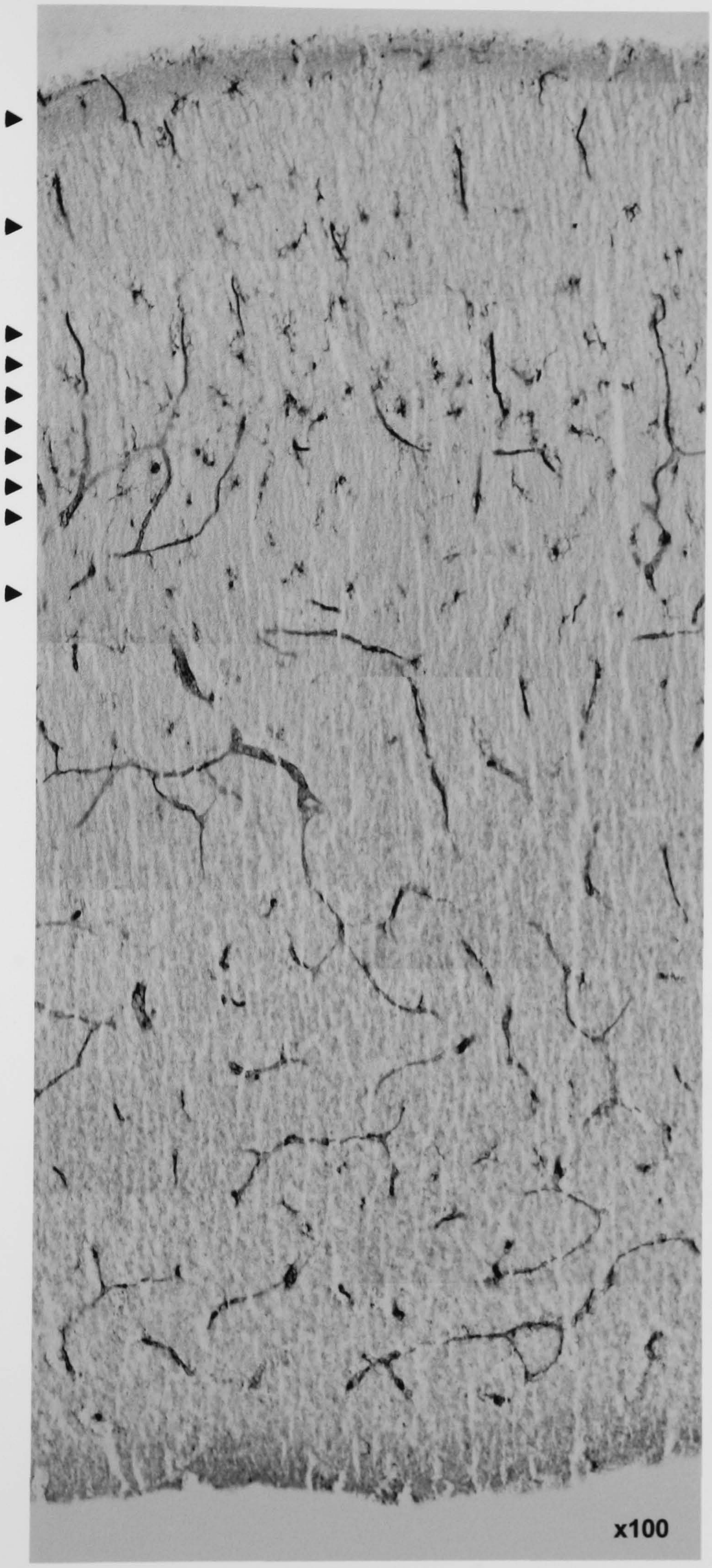


FIGURE 6

Composite figure to show RCA-1 histochemistry in human foetal telencephalon at 14GW

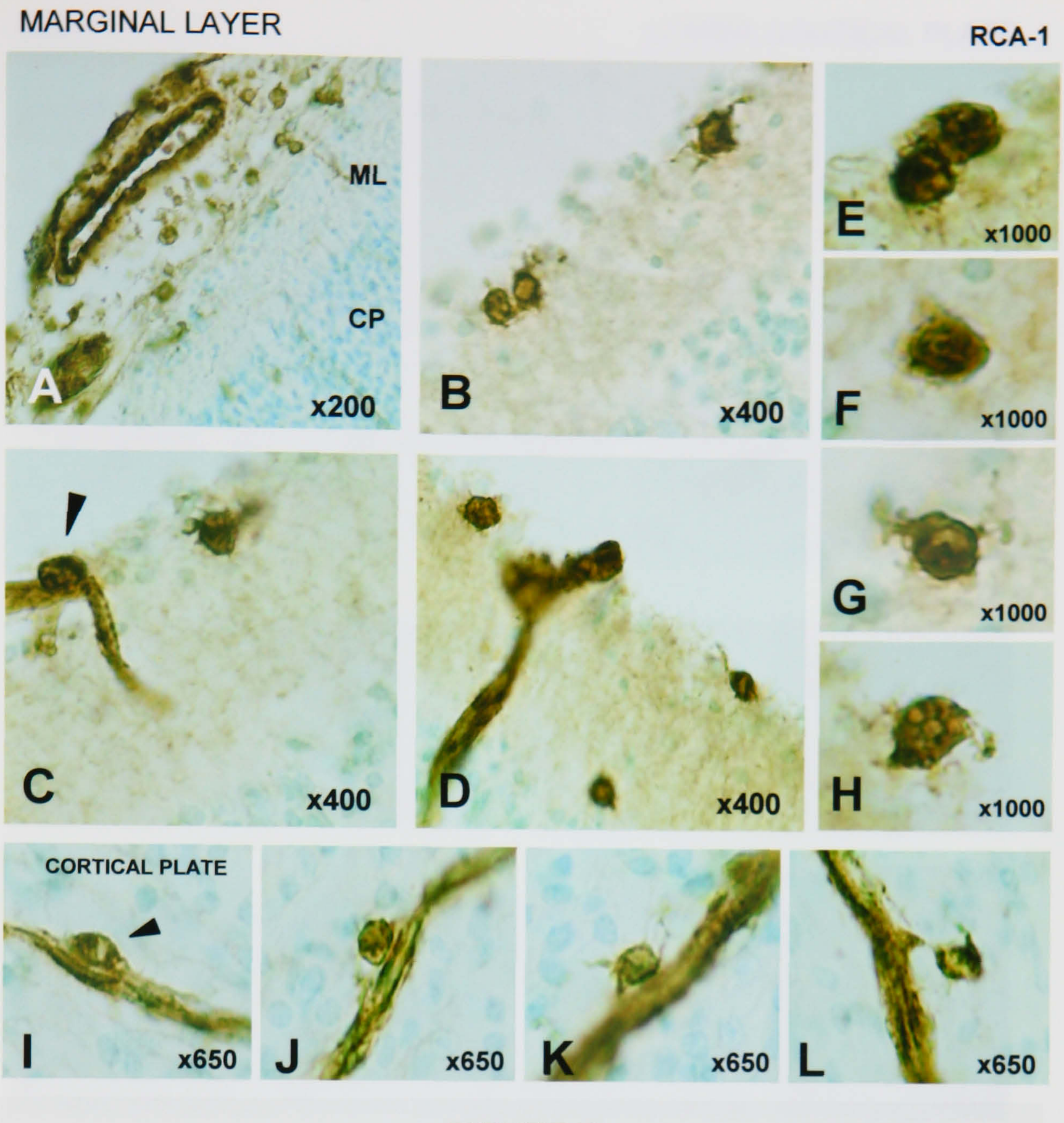


FIGURE 7

RCA-1 positive microglial progenitors in the marginal layer and cortical plate of human foetal telencephalon at 13GW

Lectin histochemistry identified blood vessels (**A,C,D,I-L**) and populations of microglial progenitors (**B-L**) within the marginal layer and cortical plate of the telencephalon between 12-14 weeks of gestation. Cells located within the marginal layer were smaller in diameter (15-20μm) and more rounded or ovoid in appearance than their CD68-immunoreactive compatriots. These cells were occasionally found in pairs (**B,E**), occurred preferentially within the marginal layer (**B-D**), and were clearly associated with the parenchymal aspect of blood vessels that penetrated the cortical plate (**I-L**). RCA-1 positive cells located *within* blood vessels could not be clearly demonstrated at this gestational age, but instead appeared to attach to the ablumenal surface of blood vessels (**C,I arrowheads**) and progressively detached from radial vessels (**J-L**).

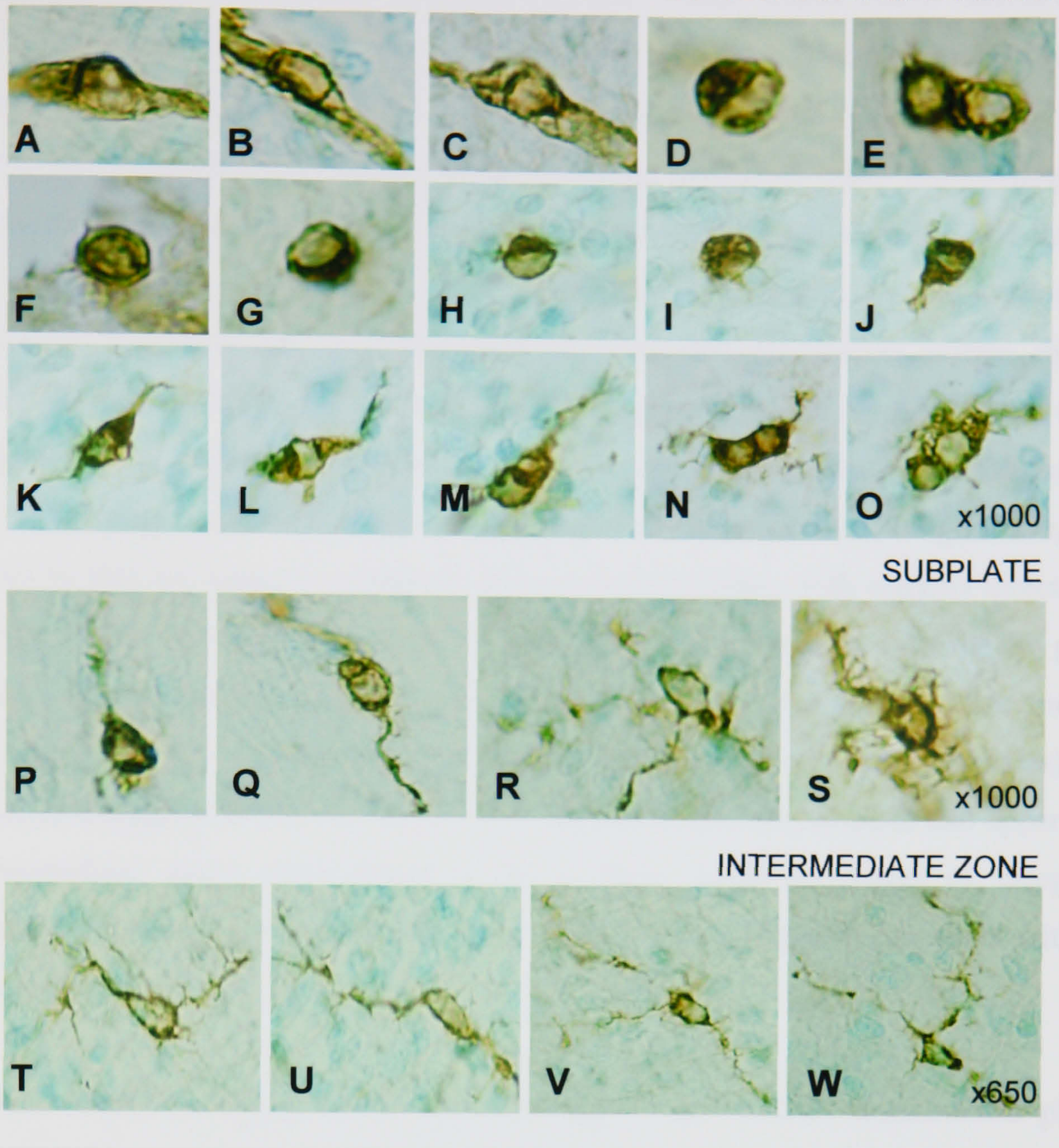


FIGURE 8

Morphology of RCA-1 positive microglial progenitors in the lower cortical plate, subplate and intermediate zone of human foetal telencephalon, 13-14GW

Examples are presented here of the morphological varieties of RCA-1 lectin-positive cells located within the cortical plate (**A-O**), subplate (**P-S**) and the upper aspect of the intermediate zone (**T-W**) of the developing telencephalon. RCA-1 positive cells can be occasionally identified within radial vessels that penetrate the cortical plate (**A-C**). Cells lying freely within the cortical plate are round or ovoid in shape (**D, F-I**). There is a clear morphological differentiation of these cell types in advancing from the cortical plate (rounded cells) to the subplate (transforming unipolar/bipolar) and upper aspect of the intermediate zone (early ramifying cells). A population of these cells is clearly dividing within the upper and (more frequently) lower cortical plate (**E, M-O**), prior to their differentiation within the subplate and intermediate zones (**P-W**). RCA-1 positive cells appear at more advanced stages of differentiation by 14GW within the subplate and upper aspect of the intermediate zone. They usually emit 2-3 (or more) delicate and long processes at various sites along the cell body and each of these in turn sprouts tributary processes along its length at various angles. These early ramified cells (**T-W**) have begun to resemble adult microglia more closely in their morphoplogy. Methyl green counterstain.

Lectin-labelled cells could not be detected with any clarity, *within* blood vessels during this period of gestation (12-13GW), but were frequently seen at various stages of 'detachment' from radial vessels within the lower aspect of the cortical plate (**Figure 7I-L**). Cells with progressively more ramified morphologies could be discerned when progressing from the marginal layer through the cortical plate (**Figure 8A-O**) to the subplate and immediately upper aspect of the intermediate zone (**Figure 8P-W**), indicating a progressive early colonisation, migration and differentiation of microglial progenitors within this region. Between 13 and 14GW, a few RCA-1 positive progenitors could be seen within as well as attached to the parenchymal surface of radial vessels in the cortical plate (**Figure 8A-C**). Again, those lying freely within the cortical plate were round or ovoid (**Figure 8D, F-I**), and could not be detected using conventional immunohistochemistry with markers directed against CD11b or CD64. A small percentage of these RCA-1 positive cells were in the process of dividing, whereas those which were encountered within the lower cortical plate and subplate had begun to extend fine processes (unipolar, bipolar and multipolar varieties), as they differentiated (**Figure 8P-S**). By 14GW, cells located within the subplate and upper aspect of the intermediate zones were found to be in more advanced stages of morphological differentiation, emitting progressively more delicate and elongated processes with angular side-branches (**Figure 8T-W**).

Elsewhere within the human foetal brain between 12 and 14GW, mononuclear phagocytes could be detected within the ganglionic eminence, germinal matrix and at the boundary of the matrix with the caudate (**Figure 9**), within the caudate and thalamus (**Figure 10**), and internal capsule (**Figure 11**, **Figure 12**).

<u>Ganglionic eminence/Germinal matrix</u>

RCA-1 positive cells were found in small numbers within the germinal matrix, but were particularly prevalent at the boundary between the ganglionic eminence and the caudate (**Figure 9A,B**). An occasional round cell was detected closely adherent to a blood vessel (**Figure 9C**), and there was also some indication that these cells were dividing within this region (**Figure 9F**). However, the majority of these lectin-positive cells were robust (up to 30-40μm in diameter), and showed cytoplasmic granules characteristic of macrophages (**Figure 9D,E,G-J**). Immunoreactivity with CD68 detected a smaller population of cells than with RCA-1, at the boundary between the ganglionic eminence and caudate. Nevertheless, CD68-positive cells located within the germinal matrix (of the ganglionic eminence) were more conspicuously evident than shown with RCA-1, and the majority of these were in the vicinity of vessels within the interior of the ganglionic eminence, rather than associated with the periphery (**Figure 9K-O**).

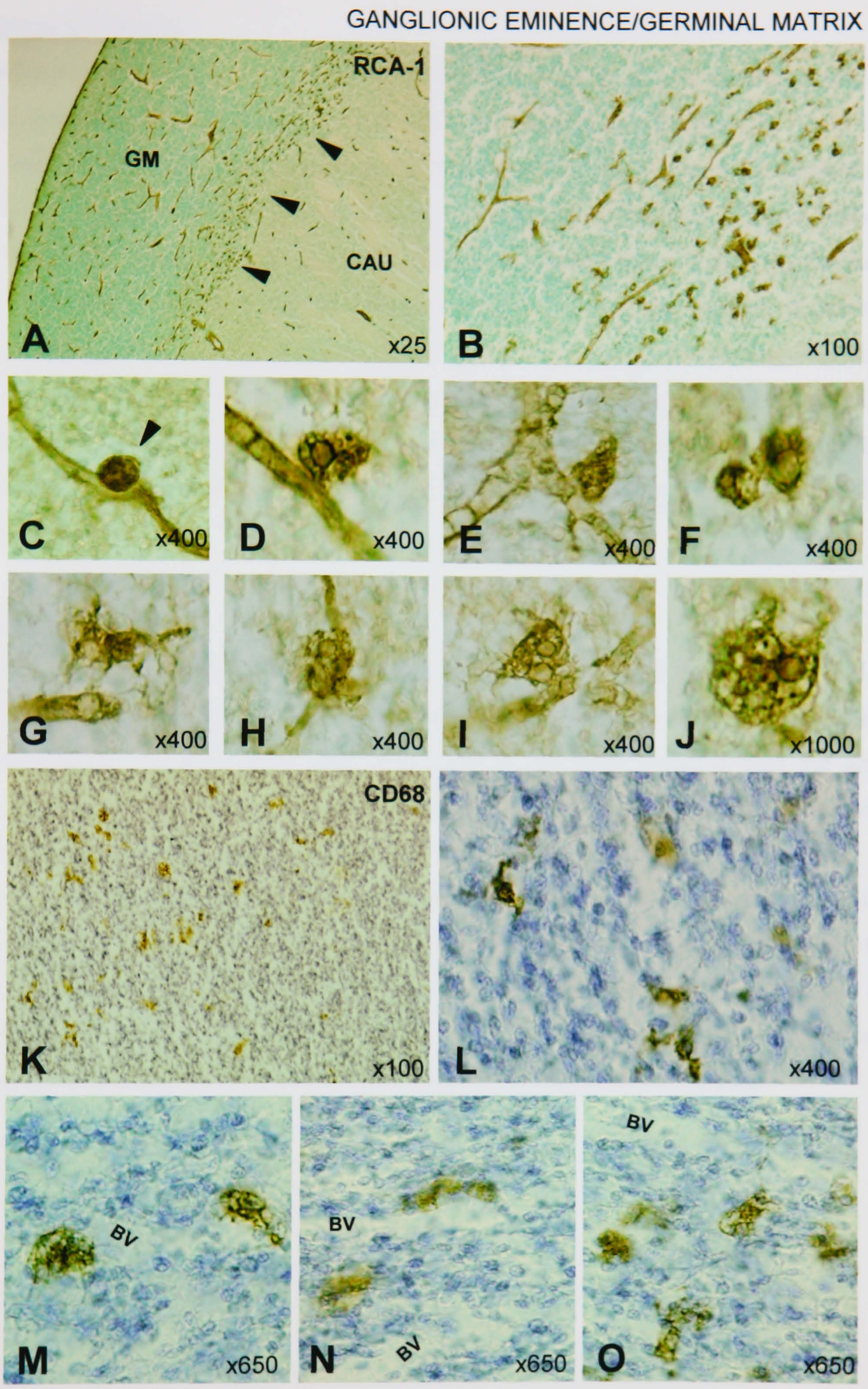


FIGURE 9

Morphology of RCA-1 and CD68 positive microglia in the human foetal ganglionic eminence and germinal matrix at 13GW

The majority of RCA-1 positive cells (**A-J**) in the ganglionic eminence and germinal matrix showed morphological characteristics typical of macrophages (**D-J**). They were particularly prevalent around blood vessels and at the border between the ganglionic eminence/germinal matrix (GM) and caudate (CAU), (**A: arrowheads, B**). Only an occasional rounded cell could be detected (**C**). CD68 positive cells (**K-O**) were more easily identified within the centre of the GM (**K,L**), in the vicinity of blood vessels (BV) (**M-O**).

Caudate and Thalamus

Likewise, within the caudate and thalamus, RCA-1 positive amoeboid cells could be seen focally distributed and associated closely with the vasculature (**Figure 10**). Their numbers were much higher within the caudate, and morphologically, those within the caudate (**Figure 10A-G**) were more robust in comparison to that found in the thalamus (**Figure 10H-L**). Not only were there fewer cells present in the vicinity of blood vessels within the thalamus, but large lectin-positive cells could be clearly identified *en passage* through thalamic blood vessels (**Figure 10J-L**). Cells passing within vessels were already morphologically similar to those that had emerged in the caudate. These observations suggest that the signals within the caudate, that preferentially recruit mononuclear phagocytes (amoeboid microglia) to this site, are already in operation at 13GW, whereas by comparison, such signals within the thalamus may be delayed.

Internal Capsule

Within the internal capsule, RCA-1 positive cells were located pasted along and within cellular columns, and closely associated with blood vessels in this region (**Figure 11**). Morphologically, most of these cells were smaller and more rounded than the amoeboid microglia (particularly those that were adhering to blood vessels, **Figure 11D**) found within the caudate or thalamus, although distinctly macrophage-like amoeboid cells could also be seen in some areas (**Figure 11F,G**). CD68 imunoreactivity within the same region (**Figure 12**) revealed an abundant population of vesicle-laden amoeboid cells, the majority of which remained close to small diameter vessels (capillaries) (**Figure 12C,E**). By evidence of their morphological characteristics and their expression of CD68, these amoeboid cells could be considered to participate in structural remodelling within the internal capsule.

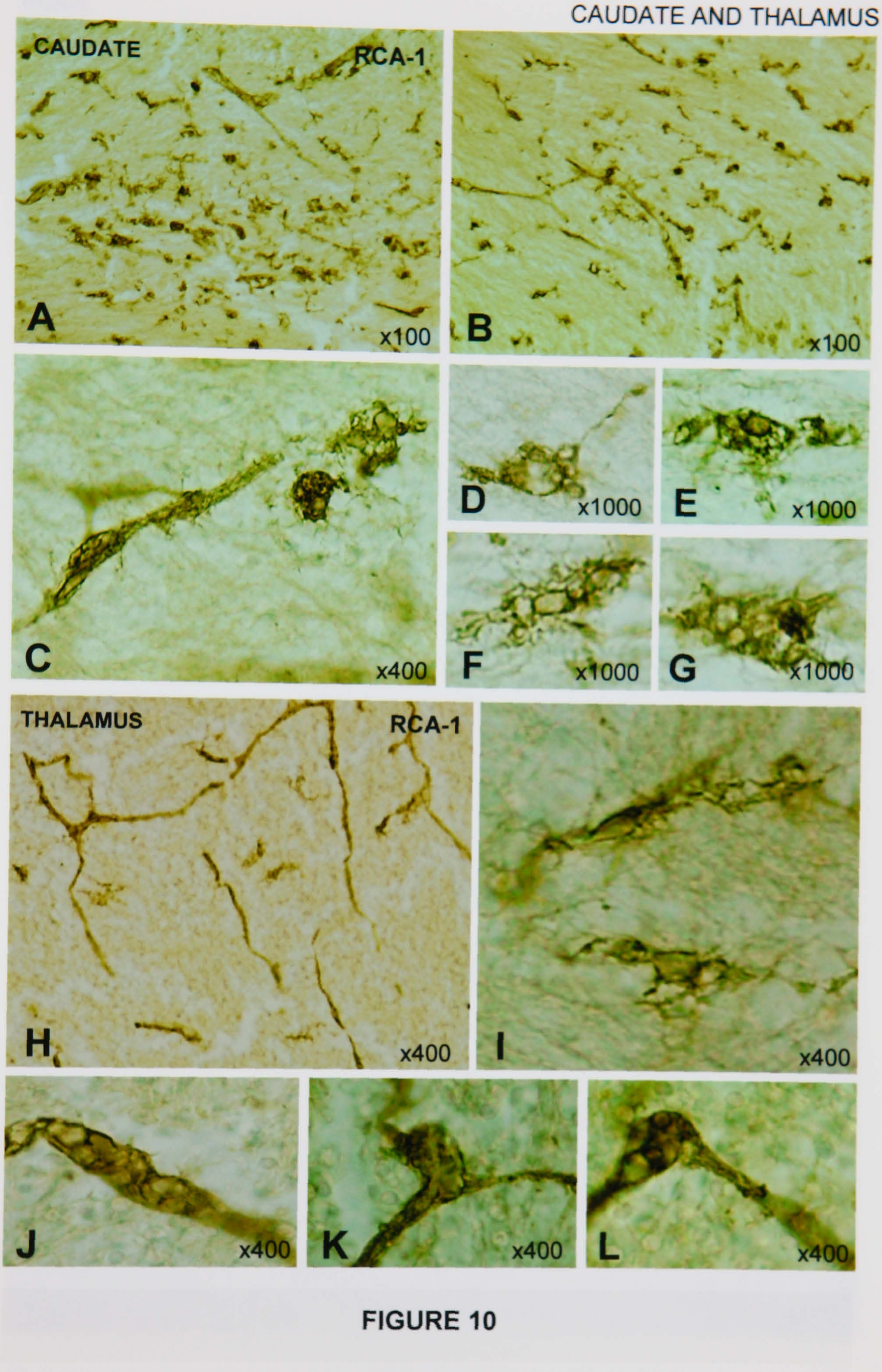


FIGURE 10

RCA-1 histochemistry to demonstrate the distribution of amoeboid microglia/macrophages and their relationship with blood vessels in the human caudate and thalamus at 13GW

(**A-G**) caudate, (**H-L**) thalamus. RCA-1 positive cells are found focally within the caudate, in the vicinity of blood vessels (**A-C**). These 'amoeboid' microglial cells show the characteristic morphology of macrophages, displaying numerous cytoplasmic granules (**D-G**). Within the thalamus, lectin-positive amoeboid cells are fewer in numbers within the parenchyma (**H,I**). In addition to these amoeboid cells, several intravascular RCA-1 labelled cells can be detected within the thalamus (**J-K**), and these already possess morphological characteristics (vesicle-laden cytoplasm) similar to those found resident within the caudate.

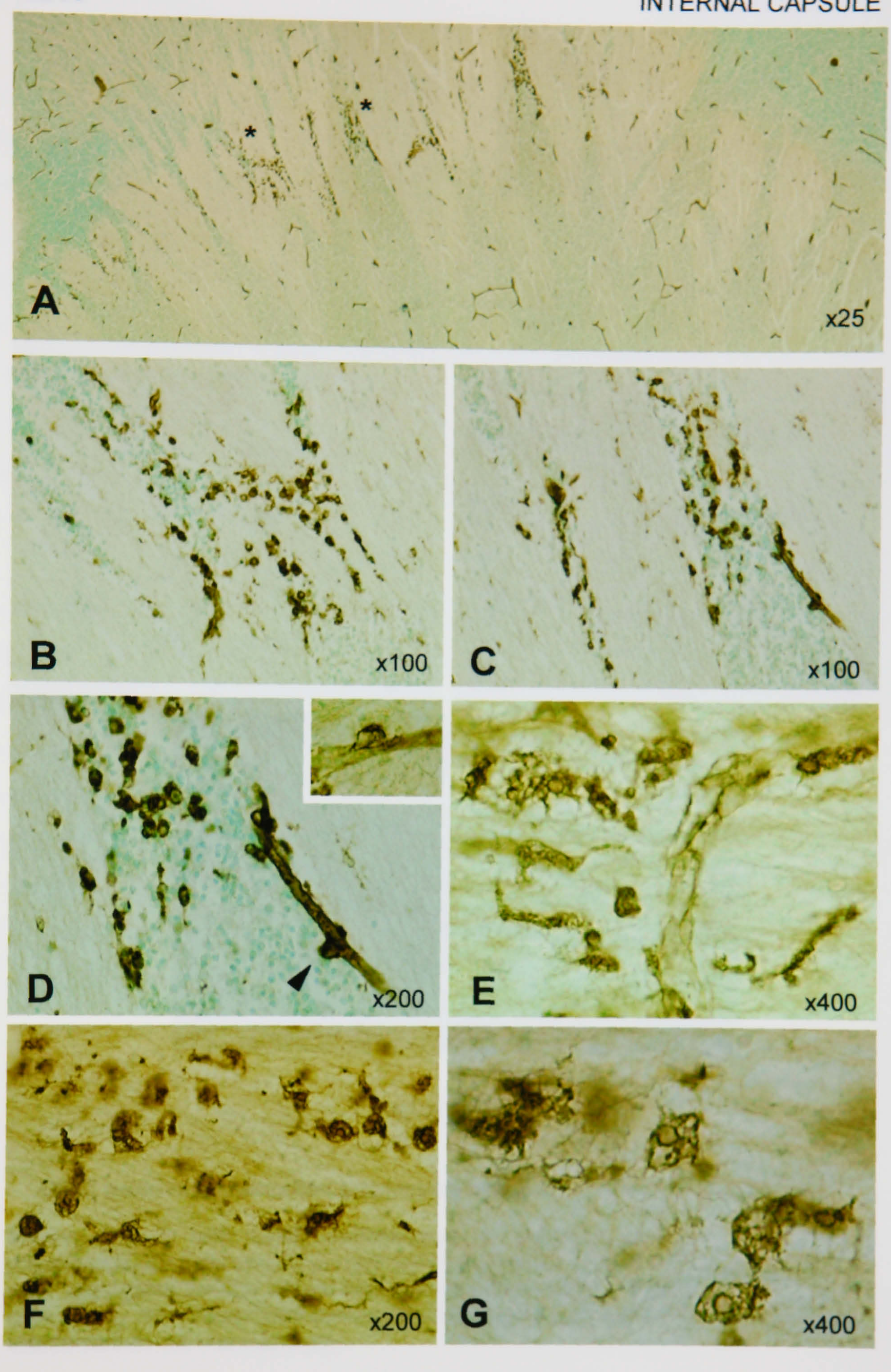


FIGURE 11

RCA-1 positive mononuclear phagocytes within the internal capsule at 13GW

RCA-1 positive cells within the internal capsule, are located along and within cellular columns (**A**). (**B,C**) show higher power photos of the respective regions designated by an asterix in (A). The majority of these cells are small and round, and several can be found pasted along vessels (**D**). Nevertheless, amoeboid macrophage-like cells laden with cytoplasmic vesicles, and resembling those within the caudate, can also be found lying freely within this transitory structure (**E-G**). Methyl green counterstain.

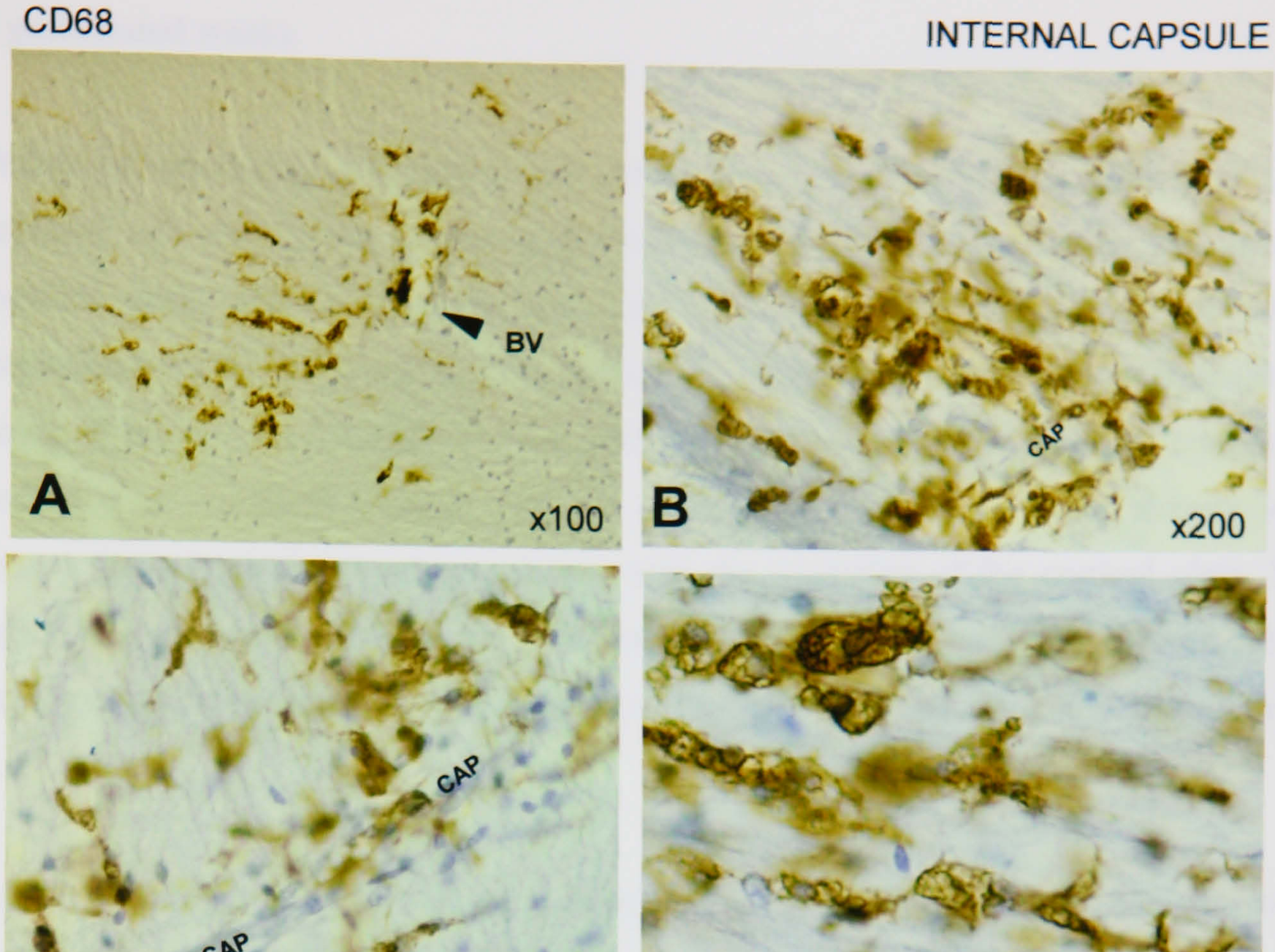


FIGURE 12

CD68 positive mononuclear phagocytes within the internal capsule at 13GW

Immunoreactivity with CD68 demonstrated an abundant population of amoeboid *macrophage-like cells already present within the internal capsule. The majority of these* were aggregated densely around blood vessels (BV) and finer capillaries (CAP). These cells closely resembled CD68 positive amoeboid cells found within the caudate. Nuclei counterstained with haematoxylin.

16-23 gestational weeks

The influx of microglial precursors was observed to be closely associated with (i) the development and maturation of the cerebrovasculature, (ii) the developing subplate, (iii) intermediate zone, (iv) ventricular zone and (v) within the corpus callosum of the telencephalon between 16 and 23GW. Colonisation, which was found to commence from the beginning of the second trimester (12-14 weeks of gestation) as shown above, continued to proceed in a co-ordinated manner, and by 22GW, foetal microglia were already widely distributed throughout the developing brain, with the exception of the cortical plate. Foetal microglia located below the cortical plate (within the subplate), adopted a more ramified morphology and downregulated phenotype, resembling adult forms more closely than in other areas.

The distribution of RCA-1 lectin positive microglia in the telencephalon between 16 and 22GW is indicated on the schematic diagrams shown in **Figure 13**. Original annotations that accompany this representation are shown in **Figure 14**. The distribution profiles for foetal microglia in these drawings represent actual regional profiles in the corresponding serial sections taken at each level per time point. **Figure 15** shows the regional distribution of foetal microglia immunolabelled with a cocktail of mononuclear phagocyte markers (CD11b:CD45:CD64:CD68) in one entire hemisphere from a foetus at 19GW. The general distribution of microglia has been plotted on representative sagittal sections (shaded in blue) and presented here, progressing from the medial to lateral aspect.

RCA-1 intensely labelled blood vessels, amoeboid microglia and macrophages in the choroid plexus and foetal microglia in the parenchyma between 16 and 23GW. However, the intensity in staining of foetal microglia toward the end of this period gradually decreased, particularly within the intermediate zone, where these cells were already sprouting finely-branched processes, as they matured (**Figure 16**). At all stages examined between 16-23GW, RCA-1 labelled microglia were more closely associated with either blood vessels or the germinal layers (VZ, SVZ) (**Figure 17**). These cells usually occurred along or within the ventricular layer, or adjacent to small-diameter blood vessels. Heavily-stained amoeboid cells were frequently observed at ventricular junctions of the choroid plexus (**Figure 17A,B**), and within transitory structures such as the internal capsule, the corpus callosum and other extensions of developing white matter (**Figures 13-15**). Microglia were less frequently detected within the marginal layer or associated within the meninges from 16-23GW.

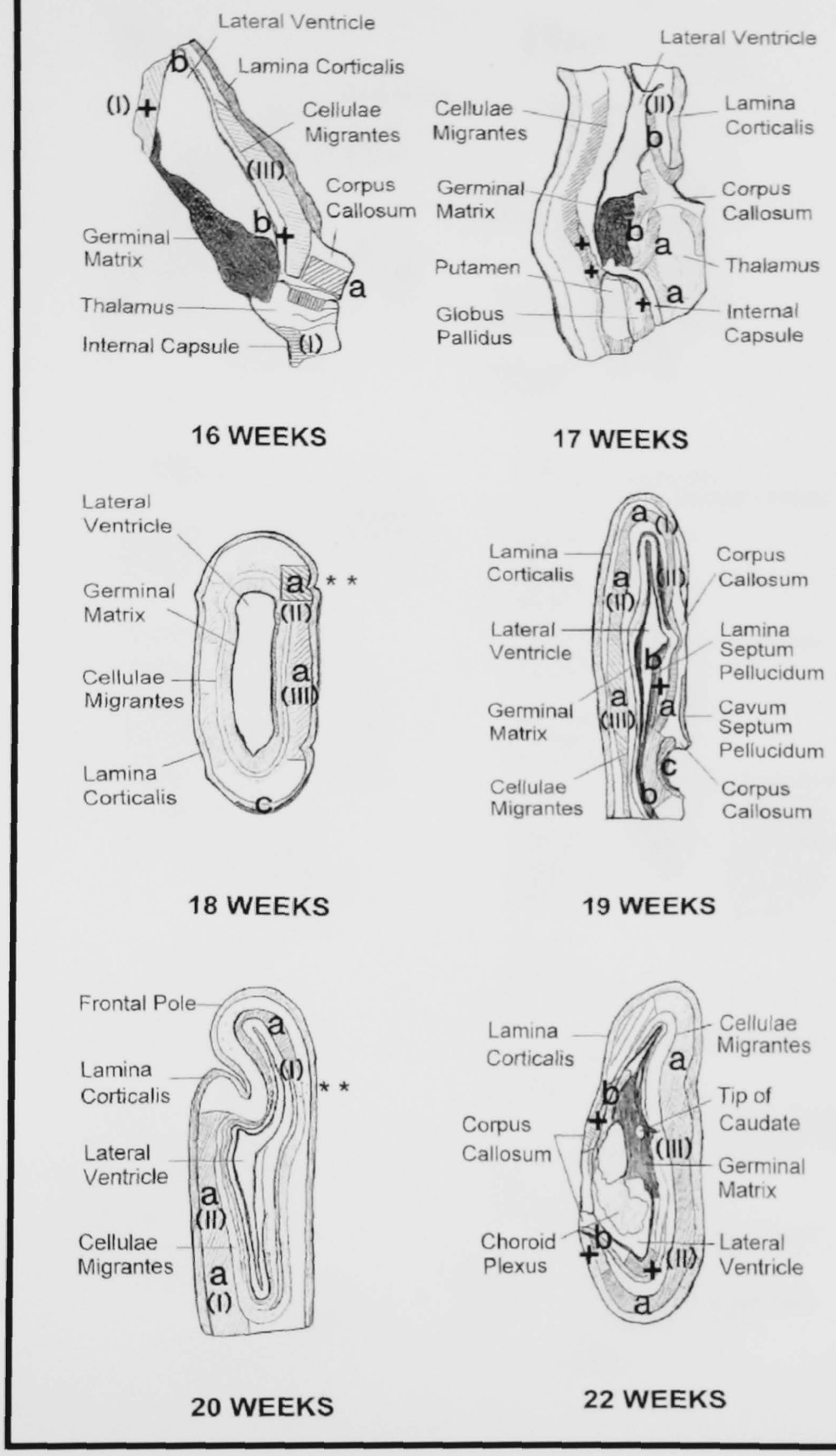


FIGURE 13

Distribution of RCA-1 positive microglia in the human foetal brain between 16 and 22GW

Schematic representations of data gathered from adjacent 20-40μmm sections. Coronal sections (16,17,18 weeks); horizontal sections (19,20,22 weeks). Distribution is indicated by hatched areas: (**a**) blood vessel associated, (**b**) ventricular associated, (**c**) meninges associated. (I) heavily stained amoeboid cells, (II) lighter stained ramified cells, (III) layered arrangement, (+) major sites of influx, (**) corresponds to the anterior corpus callosum.

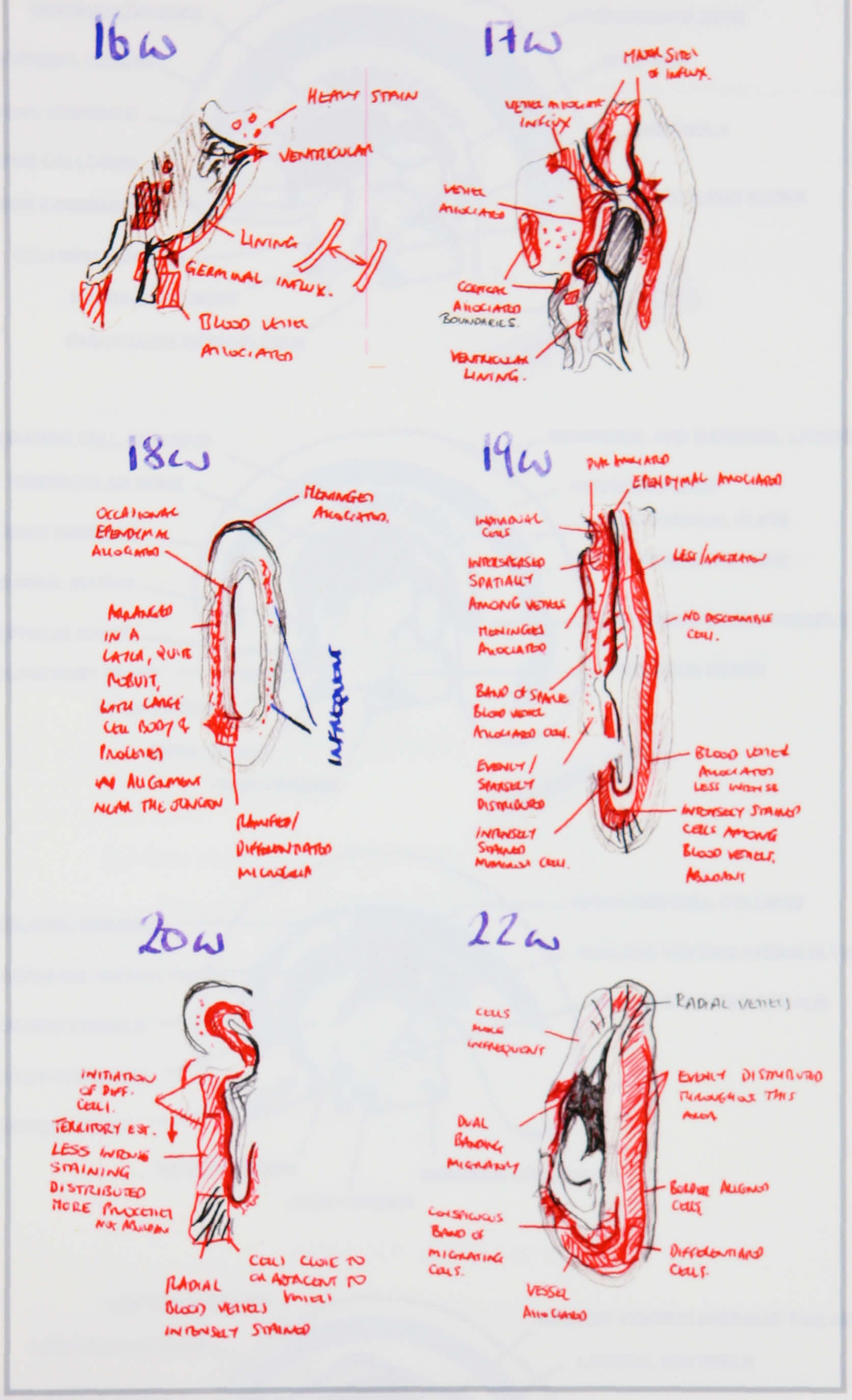


FIGURE 14

Distribution of RCA-1 positive microglia in the human foetal brain between 16 and 22GW

Original annotated illustrations from sections of the telencephalon corresponding to the schematic representation shown in Figure 13.

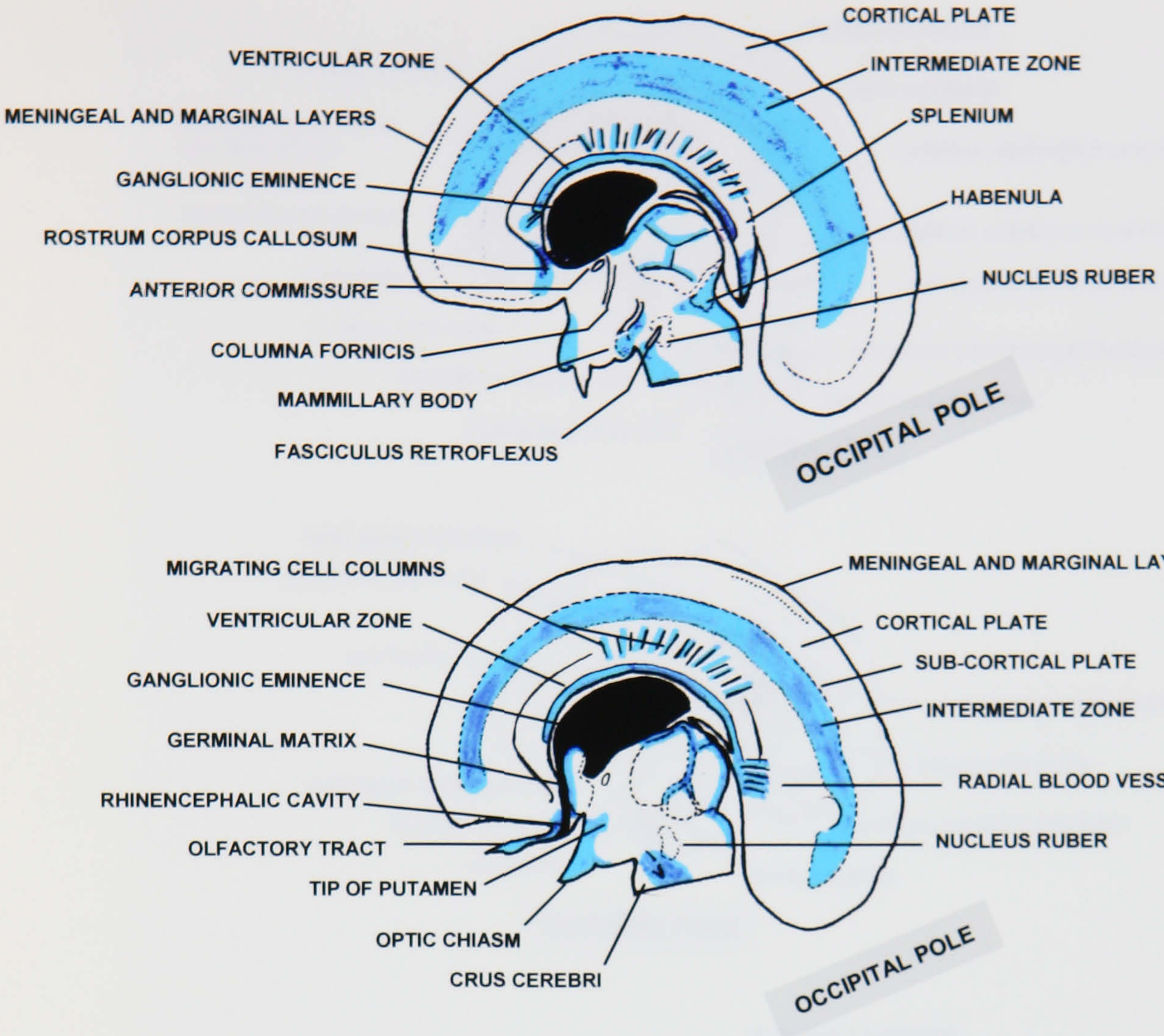


FIGURE 15

Distribution of microglia in the human brain at 19GW (sagittal view)

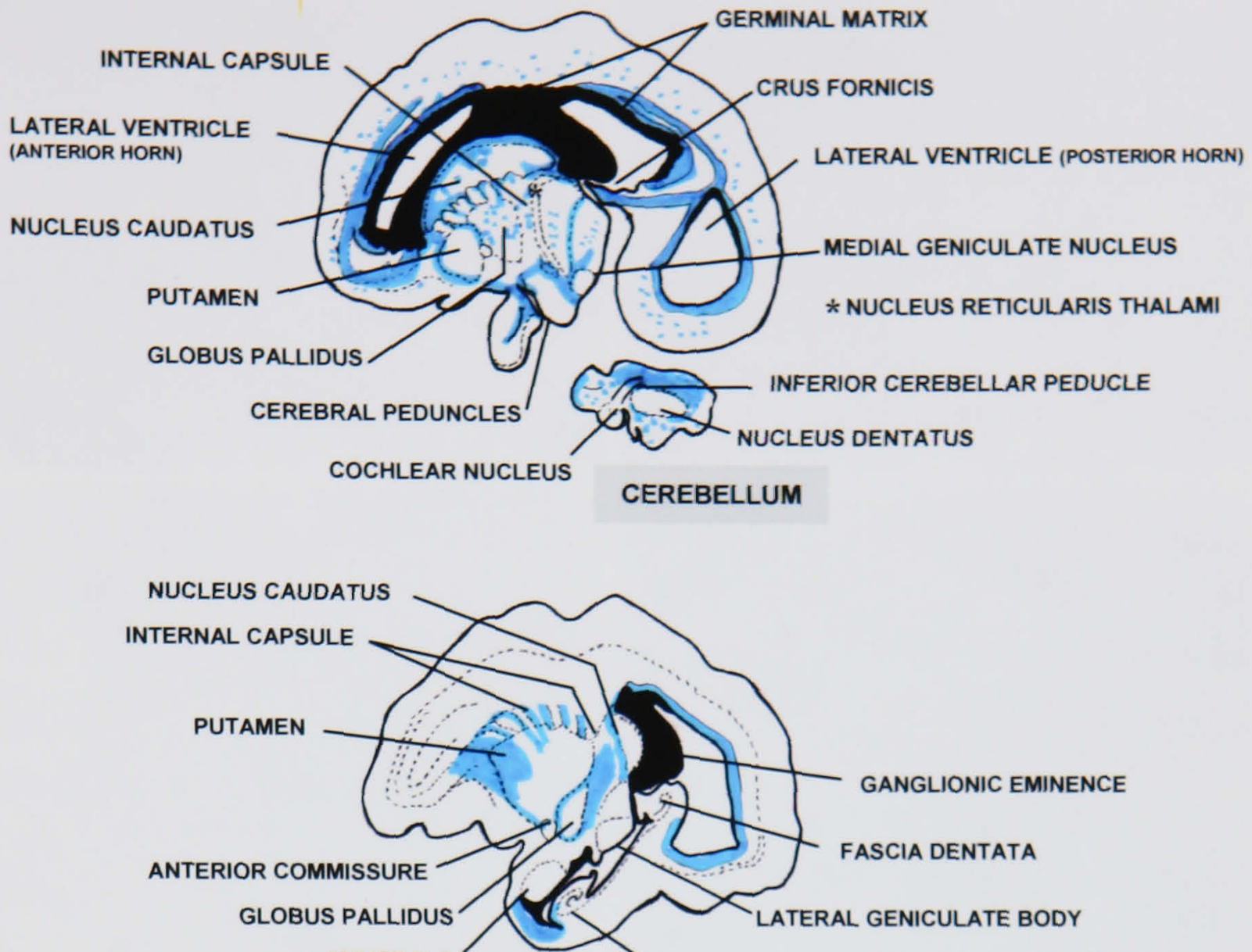


FIGURE 15 (continued)

Distribution of microglia in the human brain at 19GW (sagittal view)

Plots indicated by blue shading, represent major dispersion of microglia (there are very scarce cells in areas outside that indicated). Microglia are (i) amoeboid in the ventricular and subventricular zones, midbrain, diencephalon (thalamus), where they are often found as aggregates of cells, (ii) arranged perpendicular to the cerebral wall, in parallel arrays (associated with vimentin, CD31 and laminin immunoreactive blood vessels), (iii) evenly dispersed early ramified cells in the intermediate zone and subplate of the telencephalon. Amoeboid microglia follow tracts within the optic chiasm, olfactory tract, thalamus, crus cerebri, crus fornicis, the extent of the corpus callosum and are clearly associated with blood vessels. Amoeboid and transforming early ramified microglia are abundant at the border between the germinal matrix and the caudate (surrounding the tip of the caudate) and in the internal capsule. Vimentin positive radial glial processes and vessels appear most intensely immunoreactive in the sub-cortical plate, intermediate zone as well as within the caudate and thalamus. Microglia are clearly evident surrounding deep grey structures: basal ganglia, thalamic nuclei, habenula and within the internal capsule. They form boundaries between caudate, putamen and thalamus. However, microglial progenitors are rarely detected extravasating from 16GW onwards. Bipolar and tripolar varieties of microglia are the most common transitional morphological forms during development. Microglia identified by immunoreactivity to CD11b:CD45:CD64:CD68.

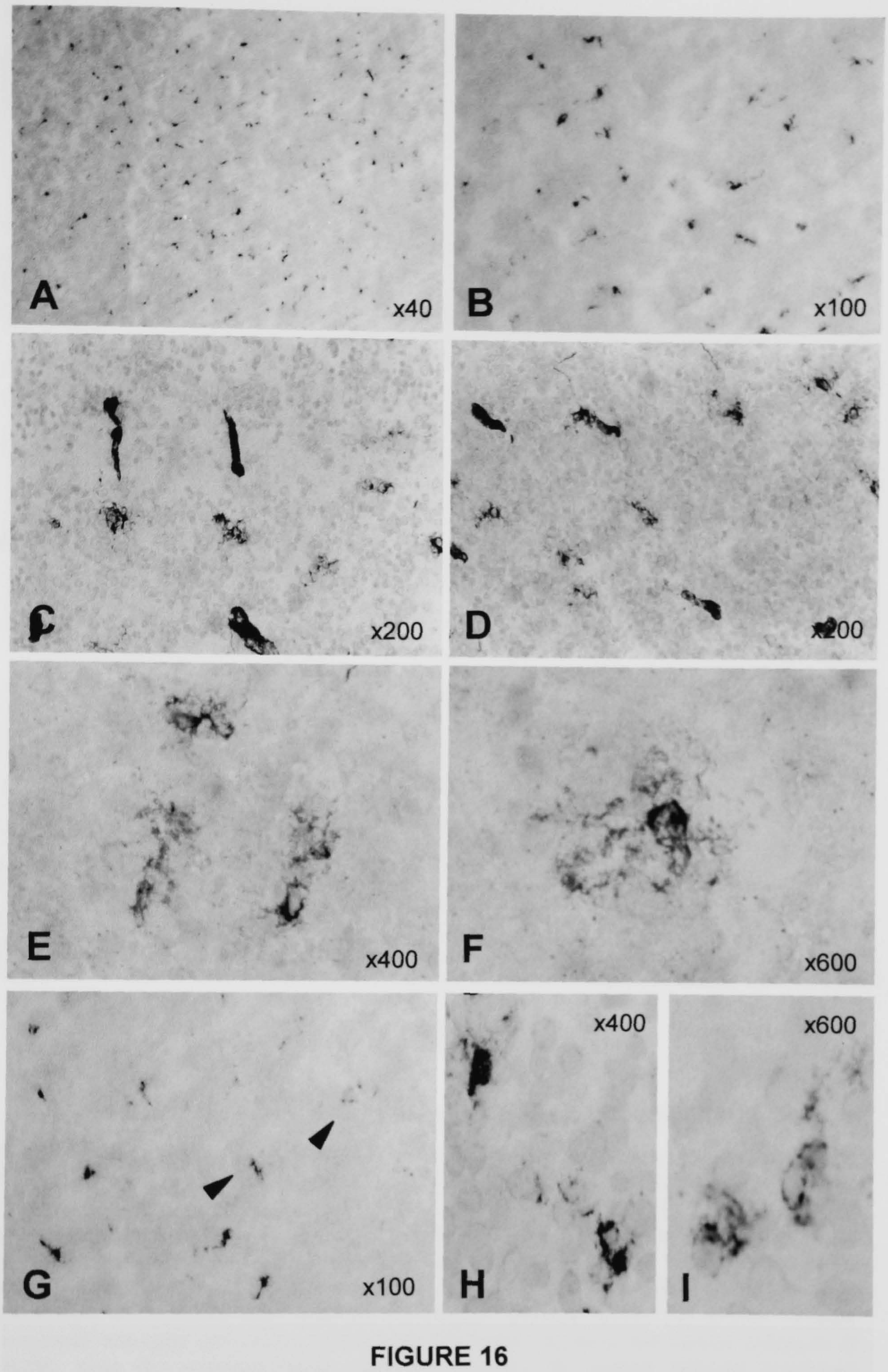


FIGURE 16

Microglia in the intermediate zone and sub-plate, 16-22GW

(**A,B**) CD11b positive microglia within the intermediate zone. (**C-D**) RCA-1 positive microglia in the subplate region, (**E,F**) Higher power figures to show the transforming early ramified morphology of RCA-1 positive foetal microglia within the subplate region. (**G-I**) CD68 (PG-M1) immunoreactive microglia in the intermediate zone at 22GW appear to downregulate this phenotypic marker when closer to the cortical plate (G, to the right of figure, cells indicated by arrowheads). (**H**) Examples of PGM-1 positive cells in the intermediate zone closer to the subventricular zone. (**I**) Examples of PGM-1 positive cells within the intermediate zone closer to the subplate/cortical plate.

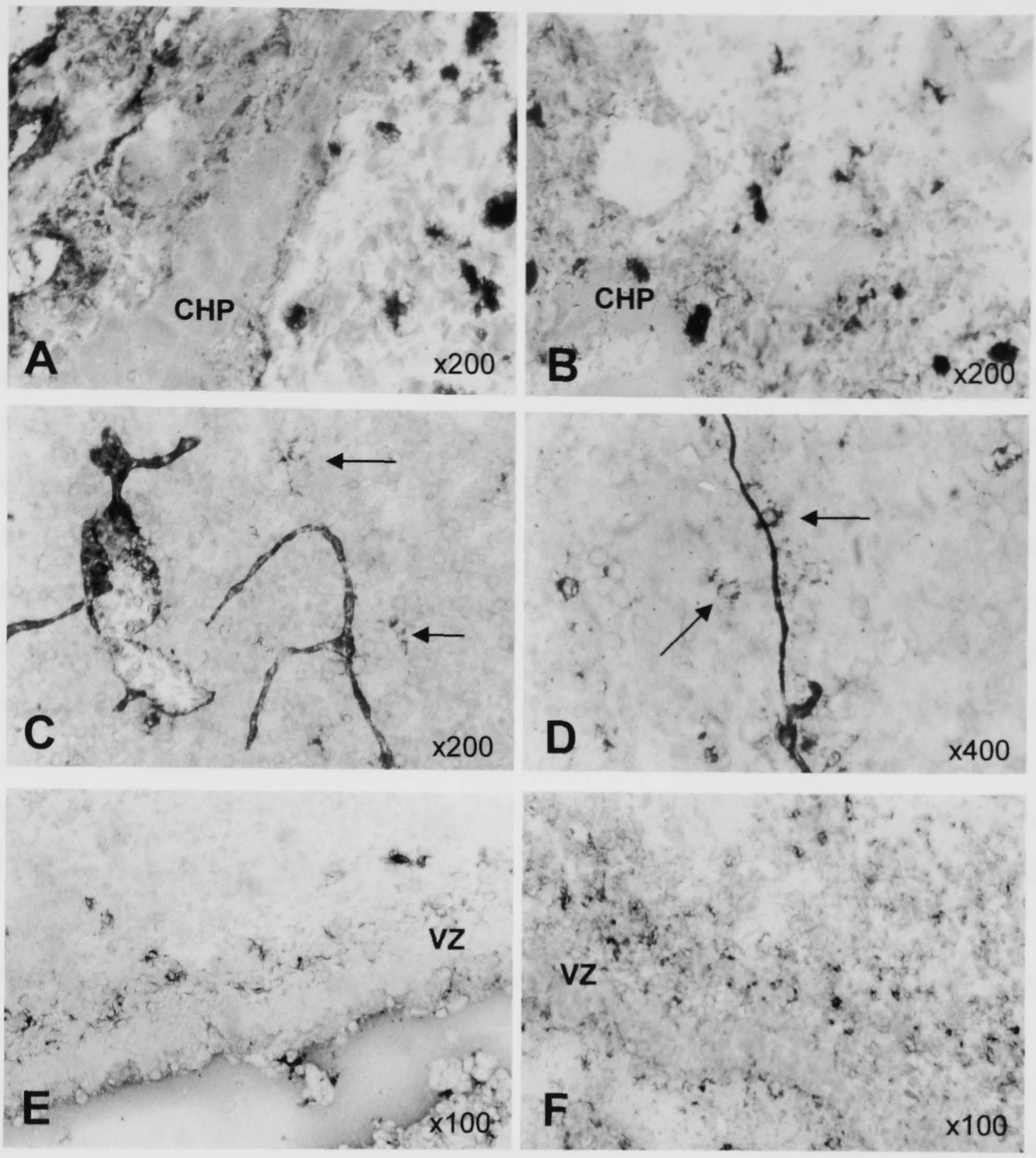


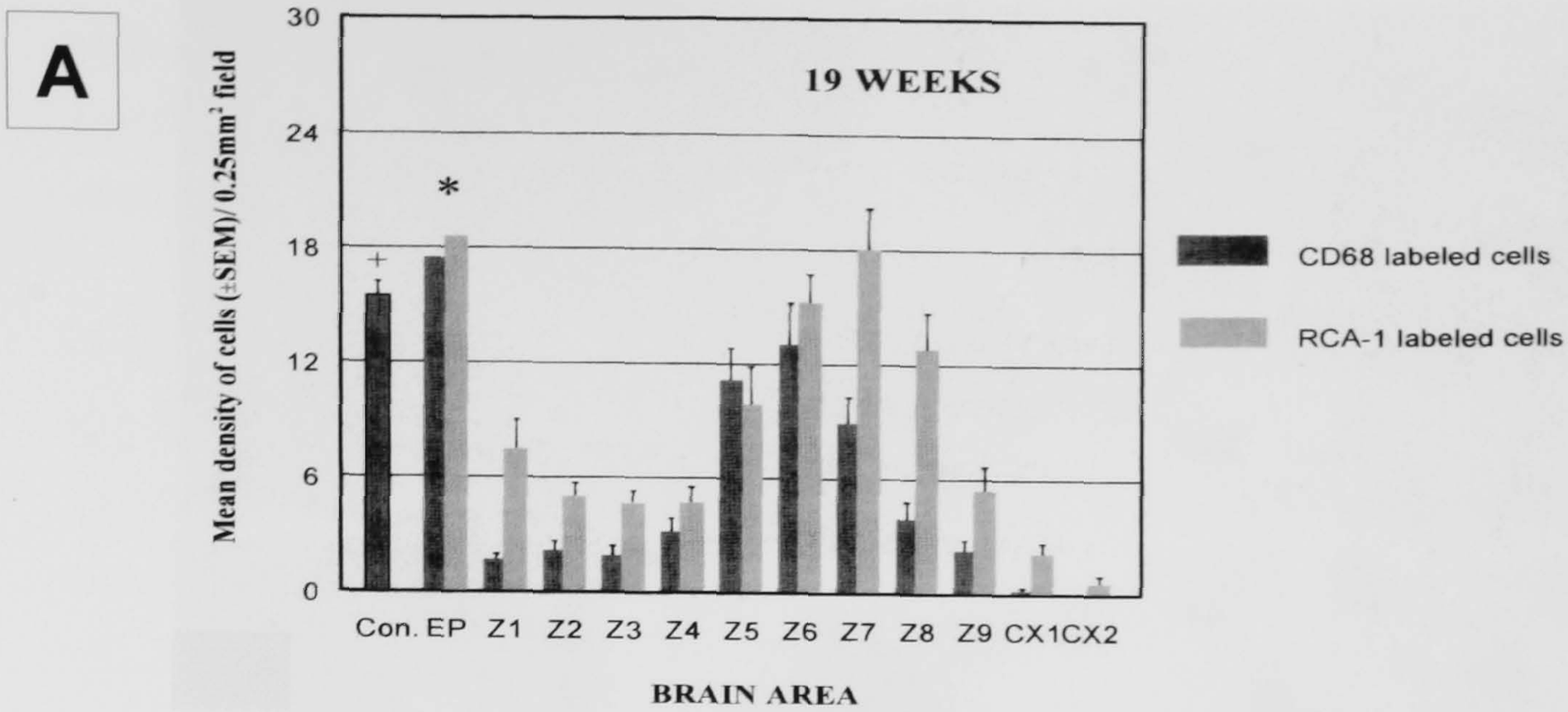
FIGURE 17

RCA-1-labelled microglia in human foetal cortex 16-22GW

Foetal microglia predominate within the following sites: (i) junctions of the choroid plexus (CHP) with ependyma lining the ventricles (**A,B**): (**A**) densely stained amoeboid cells in CNS tissue adjoining the choroid plexus at 16GW, (**B**) note the heavily stained amoeboid cells in the lining of the ventricular surface of the ependyma at 22GW where it joins with the choroid plexus. Cells within the CNS proper appear less amoeboid and may be migrating away from the site of origin. (ii) small-diameter blood vessels, particularly at transitory sites such as the corpus callosum (**C,D**): (**C**) arrows indicate amoeboid microglia associated with a small-diameter vessel in the corpus callosum at 16GW; note the adjacent larger diameter vessels, (**D**) arrows indicate amoeboid microglia associated with a small-diameter blood vessel in the intermediate zone at 19GW. (iii) subependymal zones (ventricular zone, VZ) underlying the ependymal layer of the lateral ventricles (**E,F**): (**E**) relatively few cells are stained at 19GW, (**F**) there is abundant cellular staining within the same region at 22GW. In both instances, RCA-1 labelled cells appear to migrate outwards from these original sites.

Lectin-labelled cells were also identified within and surrounding nucleated areas such as the thalamus, basal ganglia (putamen and globus pallidus) and the cavum and lamina septum pellucidum (**Figures 13-15**). RCA-1 positive cells were observed encapsulating the putamen and globus pallidus at 17GW, extending out into the internal capsule, and abundant at the apex of the junction interposed between the putamen and the germinal matrix, associated with vessels within these areas. Transforming early ramified microglia were already detectable throughout the intermediate zone at 16GW, although few in numbers. By 18-20GW, foetal microglia appeared to populate the IZ more extensively, transforming from amoeboid to early ramified forms (**Figure 16A-F**). This arrangement of foetal microglia was well-defined by 18GW and by 22GW, amoeboid cells were largely confined to the ventricular zone, subventricular zone and corpus callosum. The corpus callosum in particular, was a major site for intensely RCA-1 stained and immunoreactive amoeboid cells throughout this period. The transforming early-ramified microglia on the other hand, were distributed throughout the intermediate zone, and began to penetrate the cortical plate at various sites, undergoing maturation to adult forms. Those residing within the subplate appeared to be in a more advanced state of differentiation than cells newly recuited from the subventricular and ventricular zones. The numbers of these sub-cortical plate cells had increased by 23GW.

It was noted earlier that with transition from the ventricular zone to the cortical plate, foetal microglia were found to downregulate their immunophenotype for practically all markers examined towards the end of the second trimester, but particularly notable with CD68 (**Figure 16G-I**). In this respect, cells located closer to the SVZ were expressing these markers more heavily, whereas those situated in the intermediate zone and subplate were only weakly immunoreactive. This led to a comparison between the densities of RCA-1 and CD68 labelled microglia within the telencephalon, shown graphically in **Figure 18**. On initial inspection, CD68 and RCA-1 appeared to label different populations of foetal microglia. CD68-positive microglia were unevenly distributed up to 19 weeks, with highest densities within the ependyma and mid-intermediate zone (**Figure 18A**). By 22 weeks, CD68-positive cells were more evenly distributed. However, a new surge of RCA-1 positive cells appeared at this time, with greater densities emerging towards the ependymal/ventricular zone (**Figure 18B**). This observation could reflect either (i) the migration outwards from the ventricular and subventricular zones of a freshly-recruited population of cells towards the cortical plate, (ii) the merger of two populations of foetal microglia (those which colonise the subplate and IZ initially with cells which subsequently arrive in the VZ and SVZ), (iii) the existence of differing sub-populations of foetal microglia which differentiate regionally within the telencephalon, or (iv) the regional and transient proliferation of foetal microglia (within the VZ, SVZ or IZ).



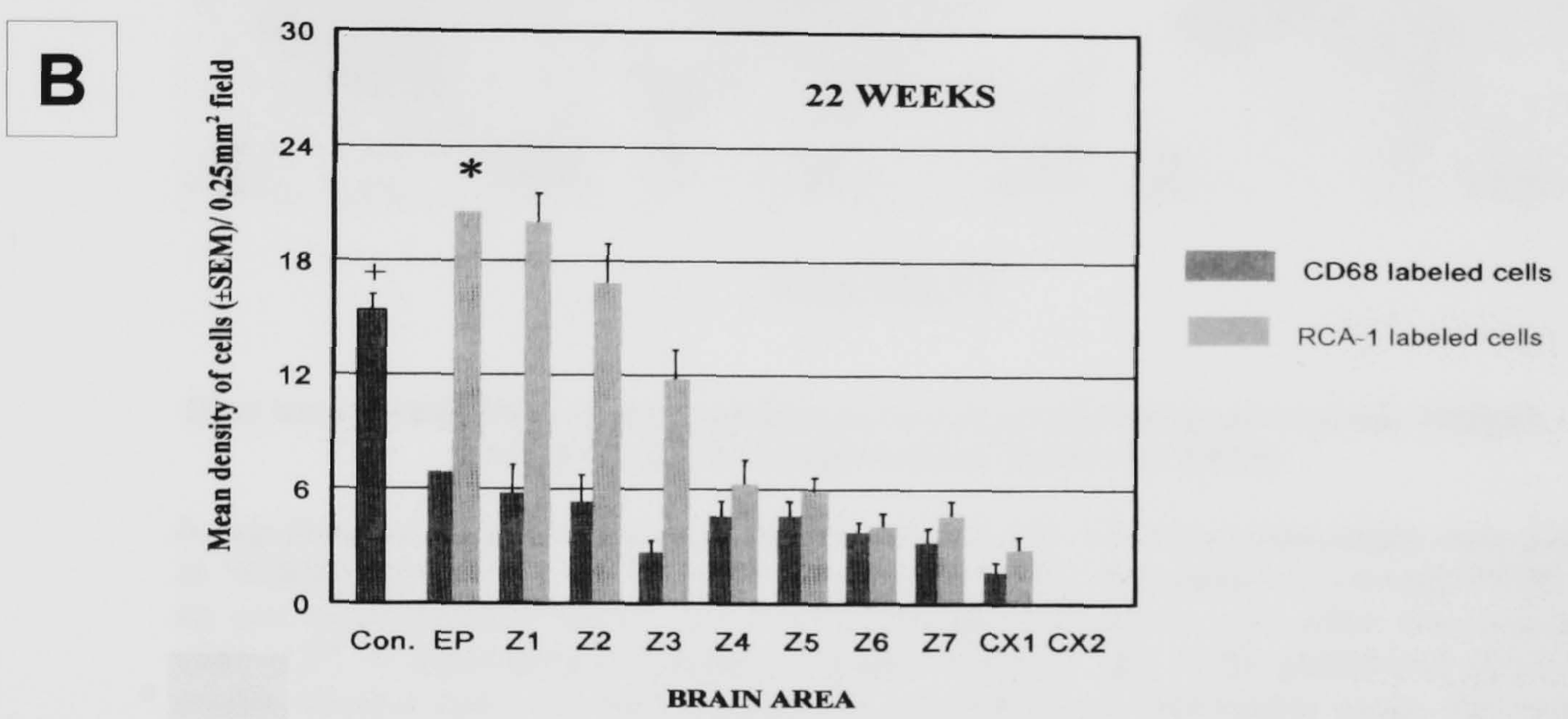


FIGURE 18

Distribution of microglia in the human foetal telencephalon at 19 and 22GW

Immunohistochemistry (CD68) and lectin histochemistry (RCA-1) were performed on 30-40μm serial cryostat sections from normal non-pathological frontal blocks, and processed for light microscopy (Rezaie et al. 1997). Control sections were taken from adult brain and stained for microglia using the Weil-Davenport silver method. All quantitative analysis was performed with a light microscope using a x20 objective, from serial RCA-1 and CD68 stained sections lightly counterstained with haematoxylin to define nuclei. Microglia were visualised using DAB (brown) or Vector-VIP (violet), and defined as positively stained cells with a distinct cell body (with/without processes). RCA-1 positive microglia were readily distinguished morphologically from cortical vessels at the section thickness employed. Data are presented as the mean density (±SEM) of foetal microglia per 0.25mm^2 area (non-overlapping fields) in 8-10 systematic parallel sweeps from the ependymal/germinal matrix layer (EP) to the cortical plate (CX). Due to the dense nature of closely-packed cells within the ependymal layer, values indicated with and asterix (*) for this area, represent the minimum number of identifiable microglia. Consecutive fields within the intermediate zone are represented as zones (Z1-Z7/Z9). Control data (+) indicate the mean density of microglia (±SEM) in 9-10 non-overlapping fields of normal adult white matter using the same defined areas and objective. Microglia were more evenly distributed within the adult brain. **A**: 19 gestational weeks. CD68 and RCA-1 labelled foetal microglia were unevenly distributed between the ependyma and cortex, with a greater density in the ependymal layer/mid-intermediate zone and minimal numbers within the cortical plate. The median values for the two distributions were significantly different (Wilcoxon signed-rank test: $n=8$, $w=0$, $p<0.05$). **B**: 22 gestational weeks. CD68 positive cells were more evenly distributed across the tissue sections. There was no significant difference in the mean density of CD68-labelled cells in fields Z1-Z7 of the intermediate zone. RCA-1 labelled a greater number of cells in what appeared to be a secondary "wave" of migration outwards from the ependyma.

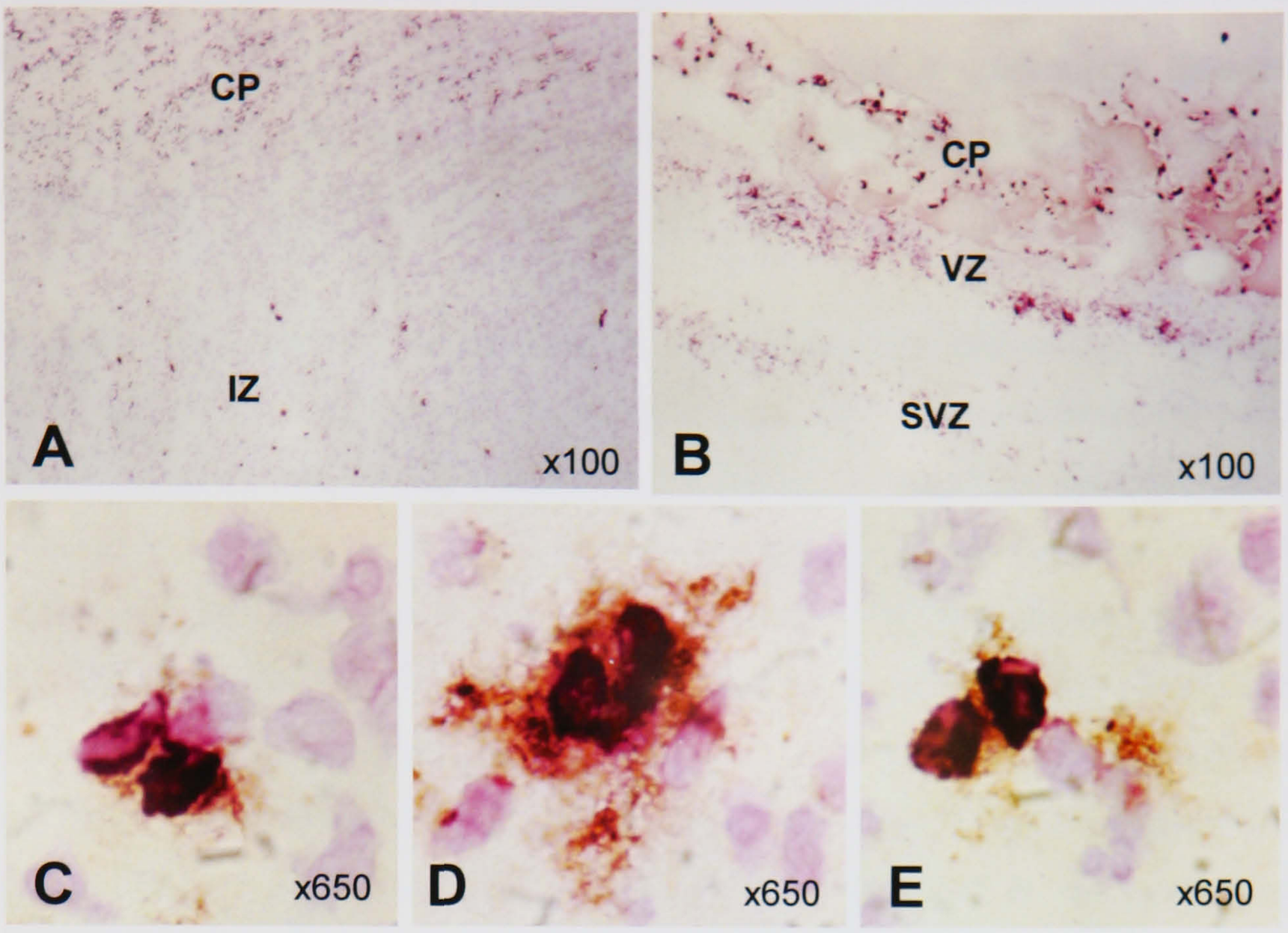


FIGURE 19

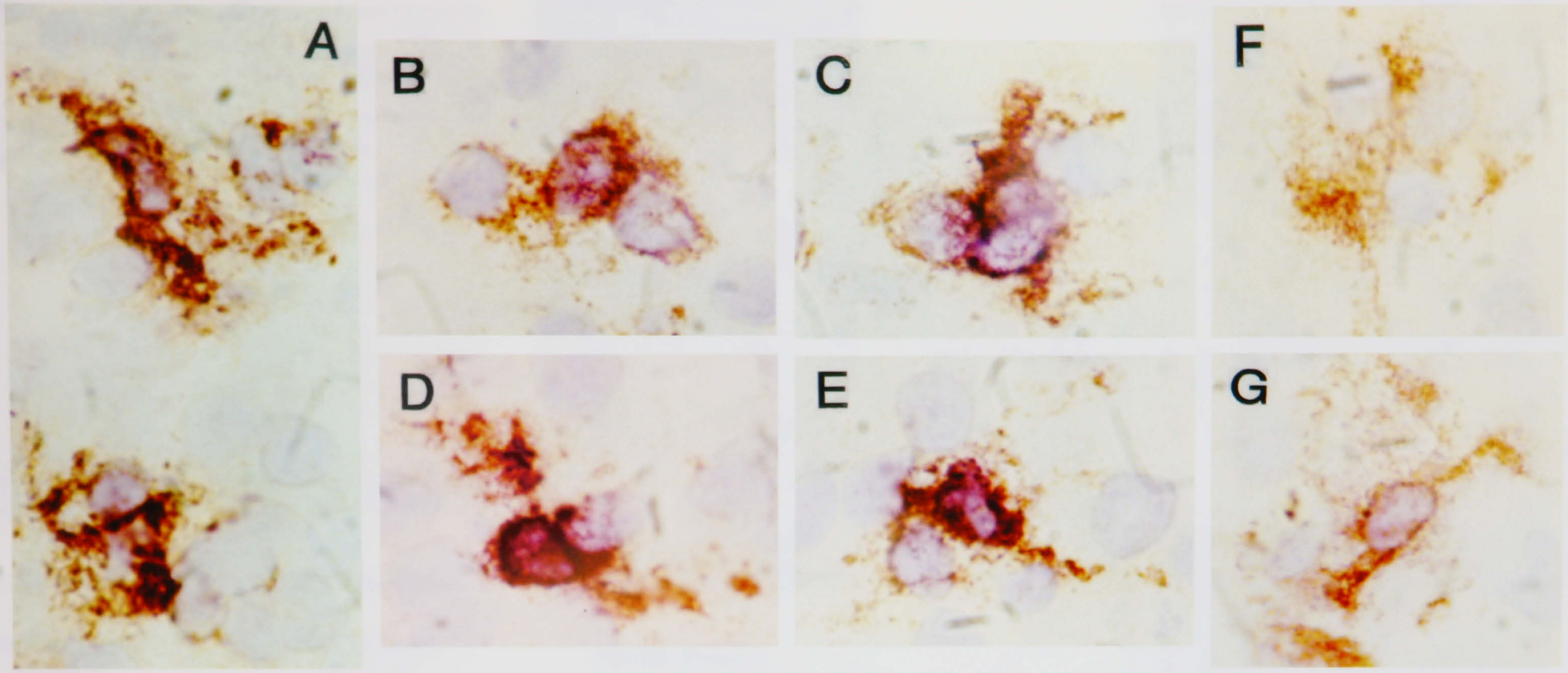
Dual immunolabelling of microglia (brown) and proliferating cell nuclear antigen (violet) in human foetal brain at 16GW and 22GW

A population of foetal microglia are actively dividing. (**A**) within the intermediate zone (IZ) at 16GW, microglia are the predominant cell population that appear to express PCNA. As one would perhaps expect, there are PCNA-immunoreactive cells within the cortical plate (CP) of developing neocortex (probably neurons). (**B**) At 22 gestational weeks, double-labelled cells are also localised to sub-ependymal (ventricular zone, VZ) and subventricular (SVZ) sites. A population of macrophages within the choroid plexus (CP) are also proliferating. (**C-E**) Higher magnification of double-labelled, dividing cells within the intermediate zone at 16GW. A: CD68 (KP-1)/PCNA, B: CD45 (LCA)/PCNA, C-E: CD68 (KP-1)/PCNA.

With respect to the latter hypothesis, an immunohistochemical assessment of proliferating cell nuclear antigen (PCNA, a co-factor of DNA polymerase-δ) expression showed that a significant proportion of foetal microglia within the intermediate zone and ventricular zones were proliferating (**Figure 19**).

RCA-1 appeared to identify all populations of mononuclear phagocytes indiscriminately throughout the period of development under study, and this phenomenon confirmed previous reports endorsing the use of this marker as a useful indicator for resting and activated microglial cell populations as well as a tool for following their development (Mannoji et al. 1986, Sreit and Kreutzberg, 1987; Suzuki et al. 1988). By comparison, foetal microglia showed a decreased level of immunoreactivity for the markers CD11b, CD45, CD64 and CD68, progressively at distances away from the ventricular zone. This progression was clear both on single and dual immunolabelling of these cells **Figure 16G-I**, **Figure 20**. Foetal microglia within the ventricular and subventricular zones and in the corpus callosum demonstrated intense expression of these markers. In the intermediate zone and subplate, expression of CD64 and CD68 was highly downregulated on cells which had begun to ramify (transitional morphological varieties). Instead, these cells expressed lower levels of CD45 or CD11b alone (according to the intensity of staining) **Figure 20F,G**. The phenotype of these foetal microglia therefore appeared to be downregulated in keeping with their morphological transformation. The process of colonisation of the human foetal telencephalon by microglia during the second trimester is summarised in the schematic illustrations shown in **Figure 21**.

In view of the immunophenotypic variation of foetal microglia, a procedure was sought that would identify all immunophenotypic subpopulations, for the purpose of direct comparison with the lectin histochemical findings. The most direct approach was to identify foetal microglia using a cocktail of monoclonal (mouse anti-human) antibodies that recognise mononuclear phagocytes. Monoclonal mouse anti-human antibodies to CD11b, CD45, CD64, and CD68 were combined in solution at a dilution of 1:20-50 per reagent and applied to the tissue sections, following the standard immunohistochemical protocol described under methods. The results were impressive (**Figure 22**, **Figure 23A-D**), and the technique has subsequently been used successfully in the laboratory for identifying all subpopulations of microglia in a variety of neuropathological disorders, as well as within the developing brain. The combined immunolabelling technique revealed a similar distribution of foetal microglia to that already described with lectin histochemistry.



Subventricular zone----------Intermediate zone----------Subplate

FIGURE 20

Morphology and phenotype of human foetal microglia with progression from the SVZ to the SP

Double immunostaining with CD11b/CD68 and CD45/CD64 on cerebral cortex at 22GW. A-F: CD11b/CD68; G: CD45/CD64. **A**: intensely stained cells within the sub-ependymal area, **B-E**: foetal microglia at the boundary between germinal matrix and transitory white matter, within the intermdiate zone. Note that the cell body of these labelled cells are closely apposed to and envelop neighbouring cells with their processes. They may be in the process of phagocytosis (engulfing cells) or alternatively receiving or reciprocating cues required for cell development/differentiation. **F**: Foetal microglia in the subplate labelled with CD11b, distinctly lacking expression of CD68. **G**: Foetal microglia in the subplate/intermedate zone labelled with CD45, distinctly lacking expression of CD64. (CD11b and CD45 brown, CD64 and CD68 violet, magnification x1000).

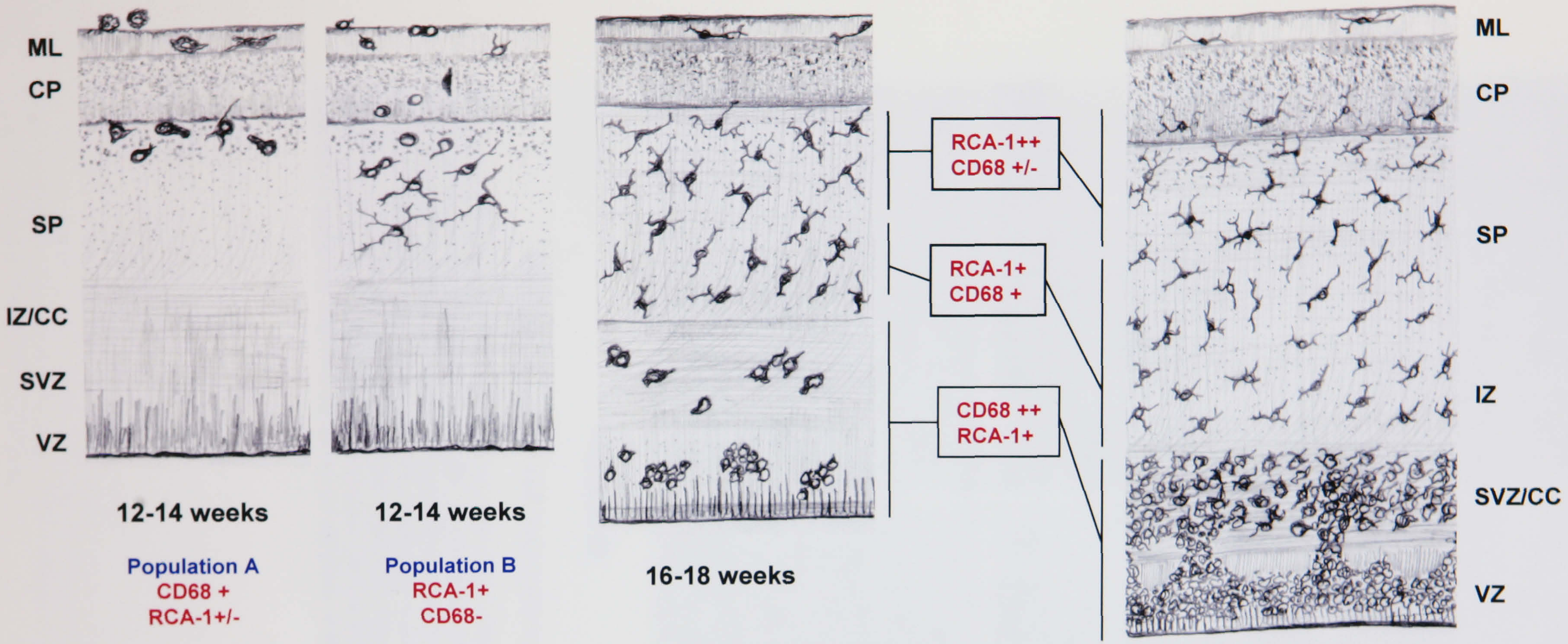


FIGURE 21

Schematic illustrations summarising colonisation of the human foetal telencephalon by microglia between 12 and 22GW

Initially between 12 and 14GW, two morphologically and phenotypically distinct populations of cells (A and B) can be detected within the marginal layer (ML), cortical plate (CP) and subplate (SP). Cell population A are amoeboid, CD68+:RCA-1+/- and reside within the marginal layer and immediately subjacent to the cortical plate. These cells resemble macrophages morphologically. Population B are small undifferentiated RCA-1+/CD68- progenitor cells within the marginal layer and cortical plate, which appear to progressively differentiate into ramified cells within the subplate. These cells do not resemble macrophages, but do resemble early forms of microglia that are found in the mature nervous system. Between 16 and 22GW, amoeboid CD68+ cells progressively accumulate additionally within the ventricular and subventricular zones (VZ, SVZ) and corpus callosum (CC), and more ramified cells can be found dispersed ubiquitously throughout the subplate (SP) and intermediate zones (IZ) of the rapidly expanding telencephalon.

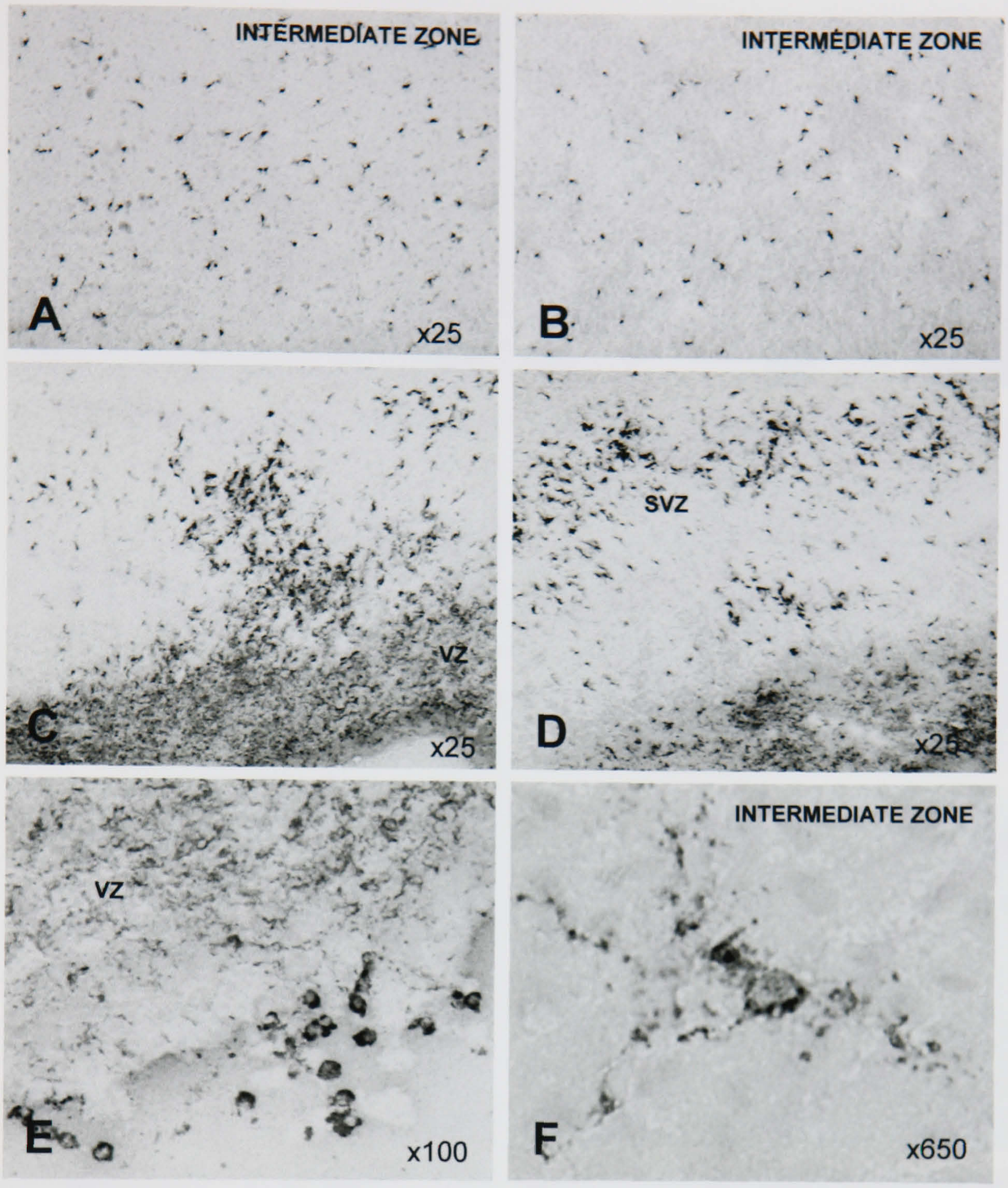


FIGURE 22

Microglia in human foetal telencephalon at 19GW

Combined immunoreactivity with CD11b:CD45:CD64:CD68 identifies foetal microglia clearly within the intermediate zone (**A,B**). In the ventricular and subventricular zones (**C,D**) foetal microglia occur as dense aggregates (corresponding to Del Rio-Hortega's 'fountains') and below the ventricular zone, supraependymal macrophages (**E**), can be detected, which are found attached to the ventricular lining of the lateral ventricles. A higher power figure of a transforming microglial cell (early ramified microglia) typical of those found within the intermediate zone and subplate, is shown in (**F**).

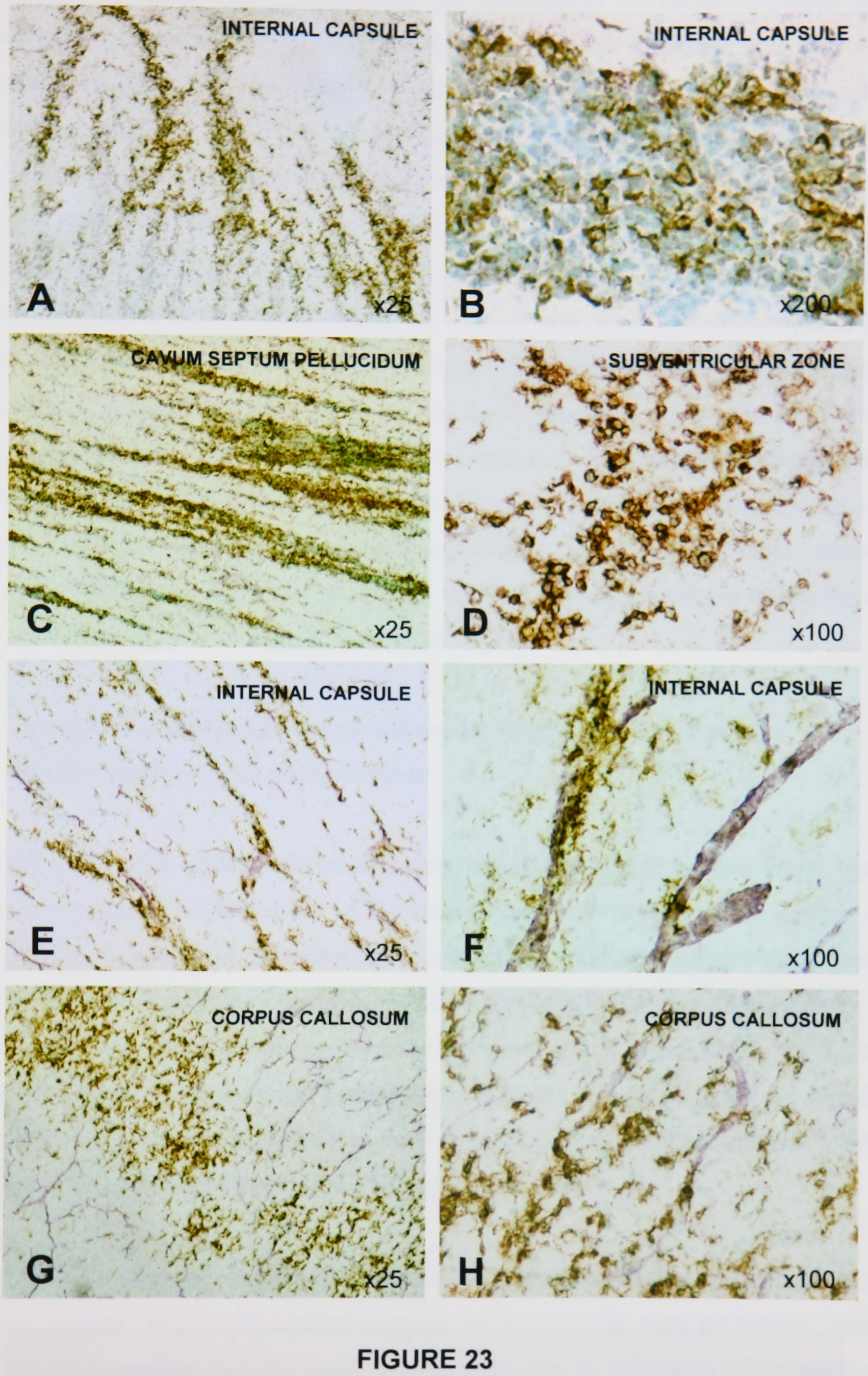


FIGURE 23

Microglia in the internal capsule, septum pellucidum, SVZ and CC at 19GW

(**A-D**) Combined immunoreactivity with CD11b:CD45:CD64:CD68 (brown) identifies foetal microglia clearly associated with cell columns within the internal capsule (**A,B**), cavum septum pellucidum (**C**) and subventricular zone (**D**) at 19 weeks. Double immunolabelling reveals the spatial relationship between microglia (brown) and laminin-immunoreactive blood vessels (violet) in the internal capsule (**E,F**) and corpus callosum/CC (**G,H**). Foetal microglia clearly associate with certain medium-large sized blood vessels in the internal capsule. Their relationship with blood vessels in the corpus callosum is not exclusive however.

In addition to more clearly identifying the populations of foetal microglia within the telencephalon (**Figure 22, Figure 23**), amoeboid cells were identified with supreme clarity elsewhere in the brain: particularly within the internal capsule and the cavum septum pellucidum, where these cells associated specifically with cell columns in linear arrangements, as already described between 12-14GW (**Figure 11A-C**), but with much greater densities (**Figure 23A**). This technique was employed to map the distribution of microglia in the entire hemisphere at 19GW, presented in **Figure 15** Amoeboid microglia were found to follow tracts within the optic chiasm, olfactory tract, crus cerebri, crus fornicis, and throughout the extent of the corpus callosum at 19GW. Amoeboid and transforming foetal microglia were abundant at the border between the germinal matrix and the caudate (surrounding the tip of the caudate) and in the internal capsule, indicating the expansion of populations seeding these areas between 12 and 14GW. Foetal microglia were clearly evident surrounding the basal ganglia, thalamic nuclei, habenula, and formed boundaries between the caudate, putamen and thalamus. The most frequent morphological subtypes of foetal microglia within these regions were amoeboid, bipolar or tripolar varieties at 19GW.

II. The spatial relationship between microglia, blood vessels and vimentin-immunoreactive radial glial processes

A close association between foetal microglia and lectin-stained cerebral blood vessels was observed within different regions of the central nervous system from the earliest stages of colonisation by microglia. A property of blood vessels in the developing human brain is their high expression of laminin, a component of the extracellular matrix, which is mainly confined to the cerebral endothelium during the second trimester (Rezaie and Male, 1999). In order to clearly ascertain a region-specific relationship between foetal microglia and blood vessels, dual label immunohistochemistry was performed using the combined markers for microglia and laminin to detect cerebral vessels (**Figure 23E-H**. **Figure 24**).

As shown in the figures, foetal microglia obviously associated with blood vessels in a region-specific manner. Within the internal capsule, amoeboid cells were profoundly accumulated around certain medium-to-large sized vessels (these may be tributaries of the middle cerebral artery) (**Figure 23 E,F**) whereas they were distinctly less numerous or absent from neighbouring vessels (**Figure 23F**). Clearly some signal from these vessels stimulated microglial adherence, migration and proliferation that was not available elsewhere on cerebral endothelium. Likewise within the corpus callosum, amoeboid microglia showed a high degree of association with some vessels, but this was not exclusive, since blood vessels on either side of the corpus callosum were devoid of these clustered cells. These observations suggested that

additional factors within this transitory structure (possibly associated with neuronal fibers), further to cues from blood vessels, were responsible for governing the highly restricted distribution of foetal microglia within the corpus callosum. The idea that foetal microglia are involved with modelling the corpus callosum, as suggested by several authors, has certain appeal. Alternatively, an equally plausible explanation could be that the corpus callosum provides a route or highway for the migration and dispersion of foetal microglia subsequent to their emigration from blood vessels. Higher magnification photomicrographs are shown in **Figure 24**, of foetal microglia and laminin-immunoreactive blood vessels within the corpus callosum. Here we can see more clearly, the close interaction between amoeboid microglia and blood vessels (**Figure 24A-C**). Occasionally, putative microglial progenitors can also be detected within the corpus callosum at 19GW (**Figure 24 D-H**). These cells are frequently detected attached to the outer wall of blood vessels, and rarely seen within. Foetal microglia do indeed appear to be dividing, as indicated (**Figure 24H**) and through immunoreactivity with PCNA (not shown).

Initial work had found vimentin to be expressed on radial glia and a small proportion of cerebral blood vessels (that penetrated the cortical plate) within the human foetal CNS (Rezaie and Male, 1999). Further investigation confirmed that immunoreactivity to vimentin was mostly confined to radial glial processes, and some cortical blood vessels within the telencephalon (**Figure 25A-C**). The most notable expression was that found in the subplate and intermediate zone, and there was a comparable lack of vimentin-positive fibers in the cortical plate. The subplate represented an area dense with a meshwork of radial glial fibers, through which passed small-diameter vimentin-positive vessels. Specifically, microglia within the subplate were intimately associated with vimentin-positive fibers, and notably lacking from neighbouring areas devoid of these processes (**Figure 25D,E**). Within the cavum septum pellucidum, parallel columnar arrangements of fibers (**Figure 25F**) likewise provided routes for the attachment of amoeboid microglia (see **Figure 23C**). Both cellular profiles and fine cellular processes could be identified within the thalamus (**Figure 25G**) and caudate (not shown), but these regions clearly lacked the structural organisation observed in the subplate and the cavum. These findings *in situ*, supported the concept of a close physico-anatomic relationship between foetal microglia and vimentin-positive radial glia and their processes in the developing human CNS.

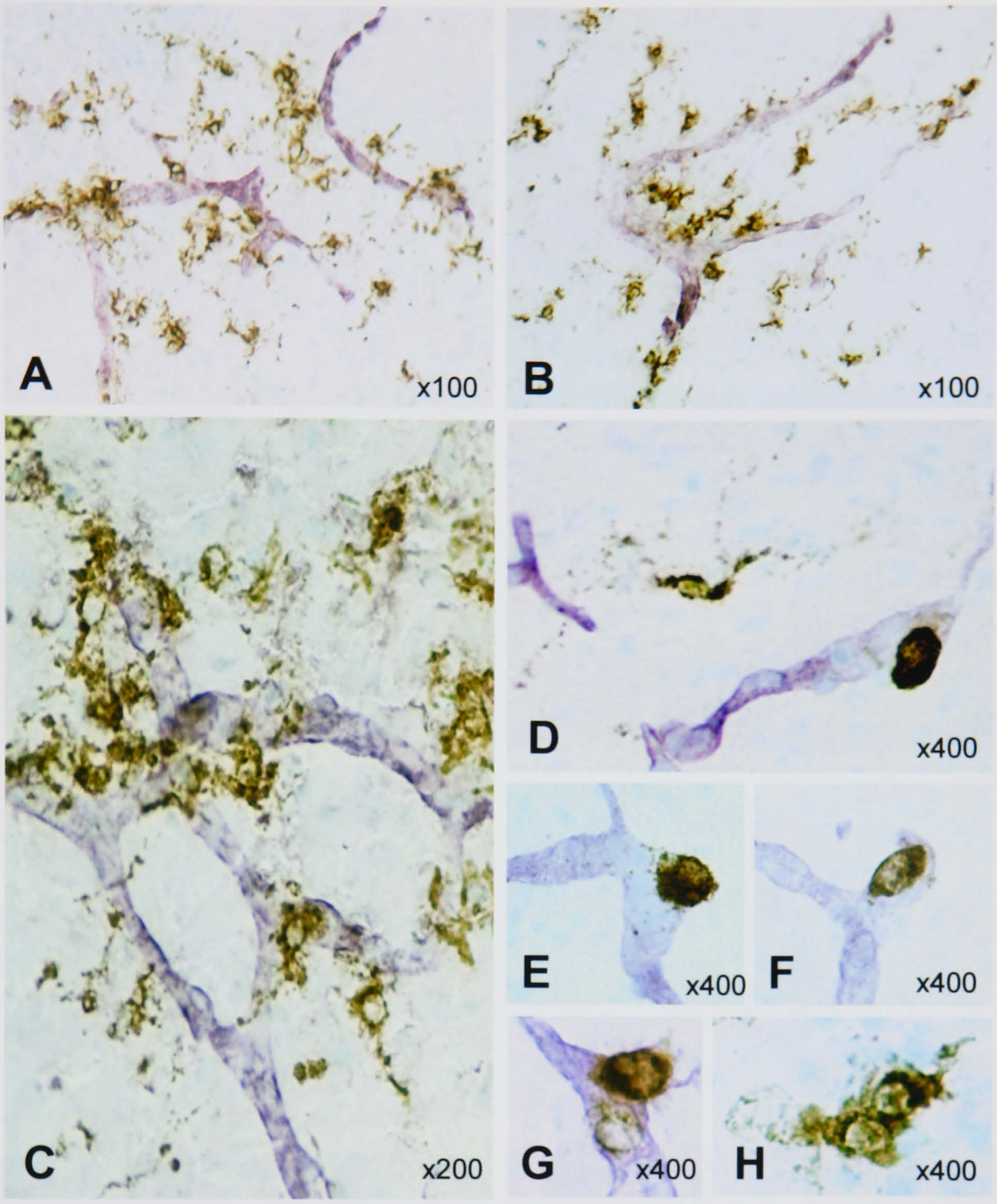
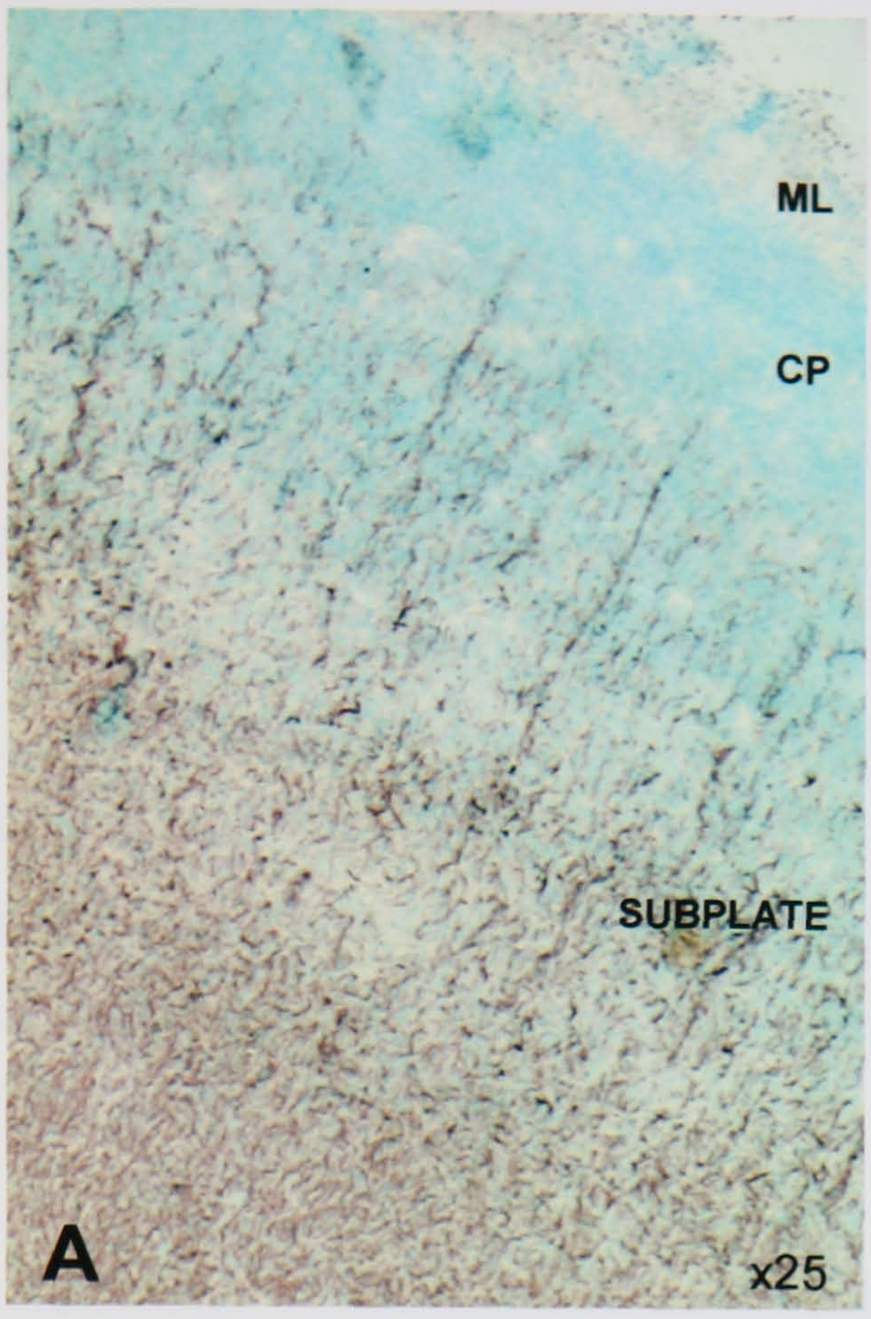
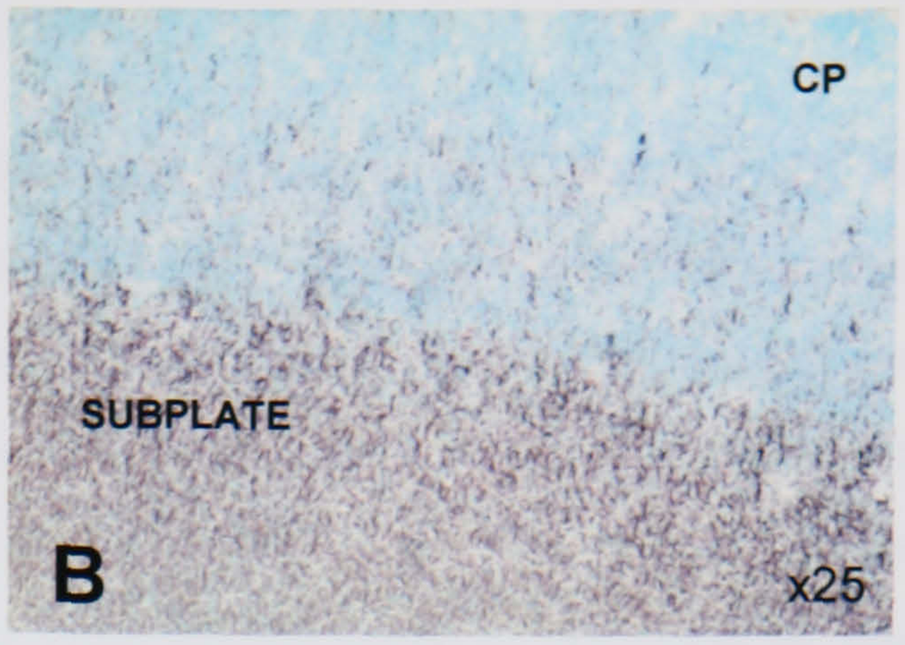


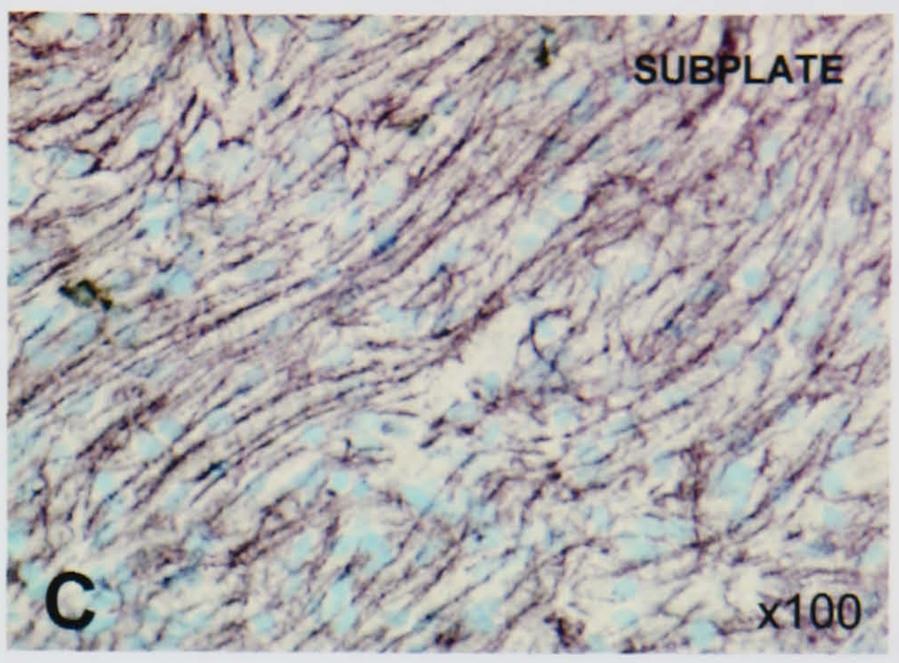
FIGURE 24

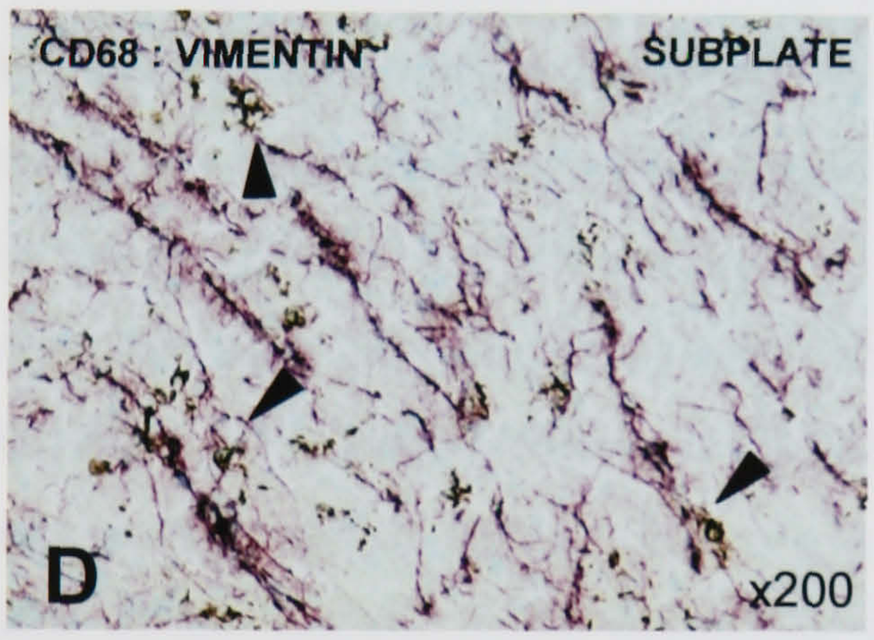
The relationship between microglia and laminin-immunoreactive blood vessels in the corpus callosum at 19GW

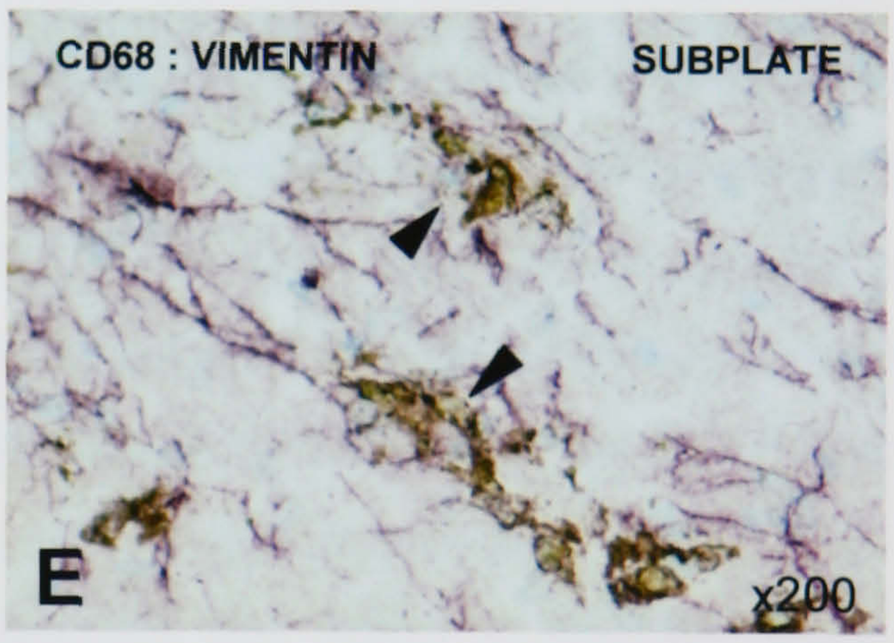
Combined immunoreactivity with CD11b:CD45:CD64:CD68 identifies foetal microglia in association with blood vessels that express laminin (**A-C**). Putative microglial progenitors can also be detected (**D-G**), including some amoeboid cells that are dividing (**H**). Microglia (brown), vessels (violet).

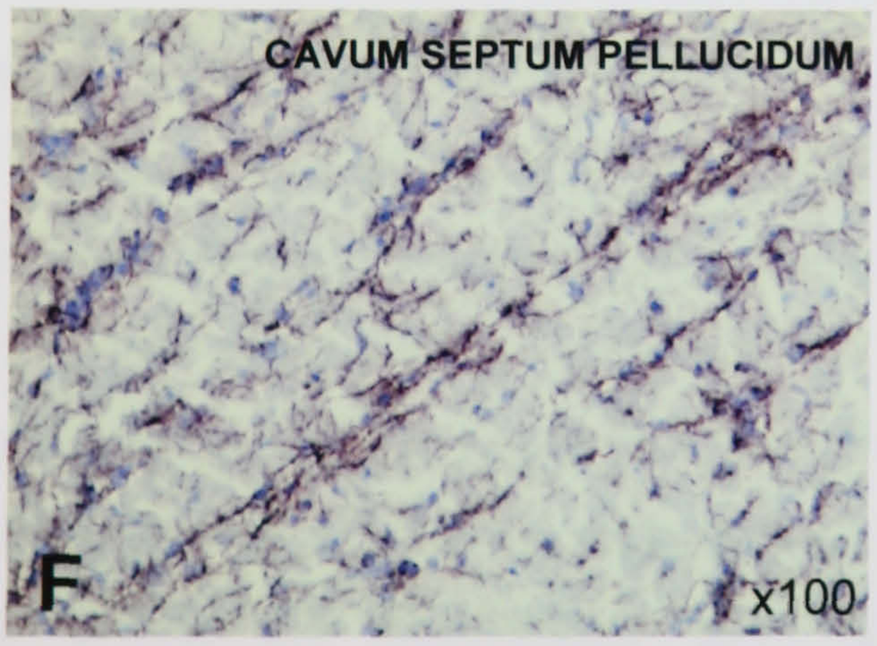












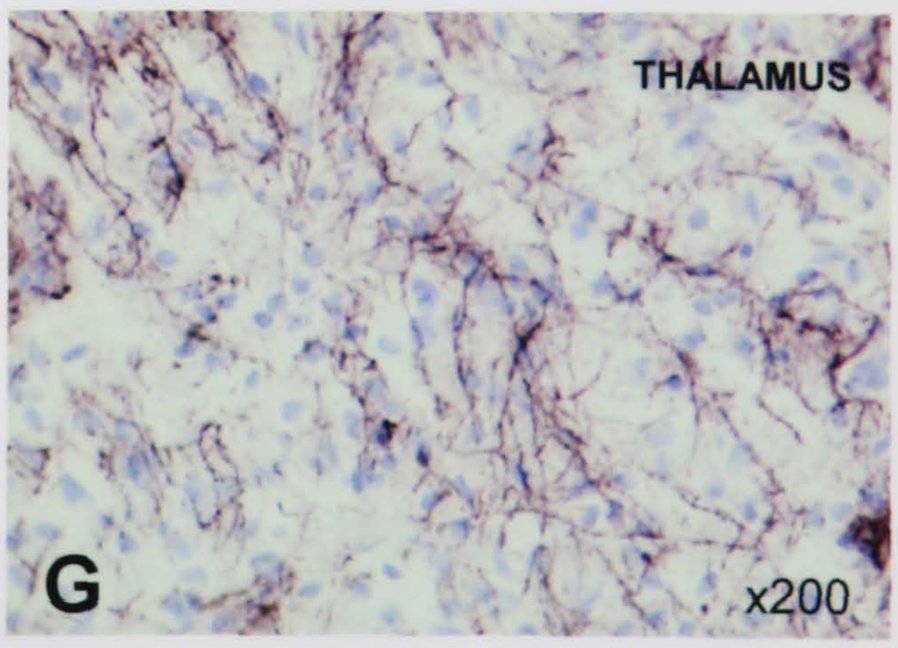


FIGURE 25

Expression of vimentin in human foetal brain at 19GW

(**A-G**) Immunoreactivity with vimentin (violet) identifies radial glial fibers within the intermediate zone of the telencephalon, in the septum pellucidum and thalamus as well as blood vessels that penetrate the cortical plate. The subplate is an area particulaly rich in vimentin-positive fibers (**B,C**), and these are arranged as undulating and tortuous forms, rather than the classic linear arrangement one encounters in other regions of the intermediate zone. Note the comparative lack of investment of the cortical plate (CP) with vimentin-immunoreactive fibers (**A,B**). Double-immunolabelling indicates microglia (brown) to be in close contact with vimentin-positive fibers (violet) in the subplate and distinctly lacking in areas devoid of these fibers (**D,E**). Likewise, radial glial fibers within the septum pellucidum are preferential sites for the accumulation of amoeboid microglia (see also figure 23C). Nuclei counterstained with methyl green.

III. Distribution of GFAP positive astrocytes in the human brain at second trimester

Having defined the temporo-spatial distribution, phenotype and morphology of foetal microglia in the human foetal brain, and their relationship with cerebral vessels and vimentin-positive radial glial fibers, the question remained as to how this distribution related to that of glial fibrillary acidic protein (GFAP)-immunoreactive astrocytes during the same period of development. A close physical and functional relationship has been established for these two cell types from studies in tissue culture and experimental work *in vivo* (Rezaie and Male, 2002). During development, the synthesis and accumulation of GFAP is considered to correspond with the morphological and functional maturation of astrocyte progenitors (including radial glia which transform into astrocytes), which also takes place within the second trimester. Thus, the expression of GFAP was next examined in a series of human foetal brains from 19 to 23GW.

The morphology of GFAP-positive cells in these normal human brains is presented in **Figures 26-31**. GFAP-positive cells in a case of hypoxic-ischeamic injury at 26-27GW, are shown in **Figure 32**, for comparison as a pathological control. GFAP was detected on progenitor cells within the ventricular and subventricular zone, where they were closely apposed to blood vessels in these germinal layers, on radial glial fibers and on tanycytes lining the ventricles at 19GW (**Figure 26**). Radial fibers and columnar arrangements of fibers and GFAP positive cell columns were identified within the cavum septum pellucidum between 19 and 20GW (**Figure 27A-C**). GFAP positive cells within the thalamus had begun to resemble more mature astrocytes morphologically, by this time, and there was clear indication that they associated with blood vessels (**Figure 27D-F**). By 21GW, cells expressing GFAP were evident interspersed throughout the intermediate zone, and those that resided within the subplate demonstrated more intense expression of this protein (**Figure 28**). Polarised cells that extended fine processes (radial glia) from the SVZ through the IZ were frequently detected, and immature GFAP positive astrocyte progenitors were common in the caudate (**Figure 28D**). Within the intermediate zone, astrocyte progenitors were found in various morphological states from round immature progenitors to cells which sprouted three or more processes and those that extended fine processes from either end of the cell body (**Figure 29**). These latter types (see **Figure 29C**) resembled transforming radial glial progenitors described by others to occur in this region. By 23GW, GFAP positive cells in the intermediate zone were undergoing morphological transformation from rounded progenitors to immature astrocytes (**Figure 30**). Surprisingly, many cells within the subplate remained in a less differentiated state even at this more advanced stage in development (**Figure 30**, **Figure 31**).

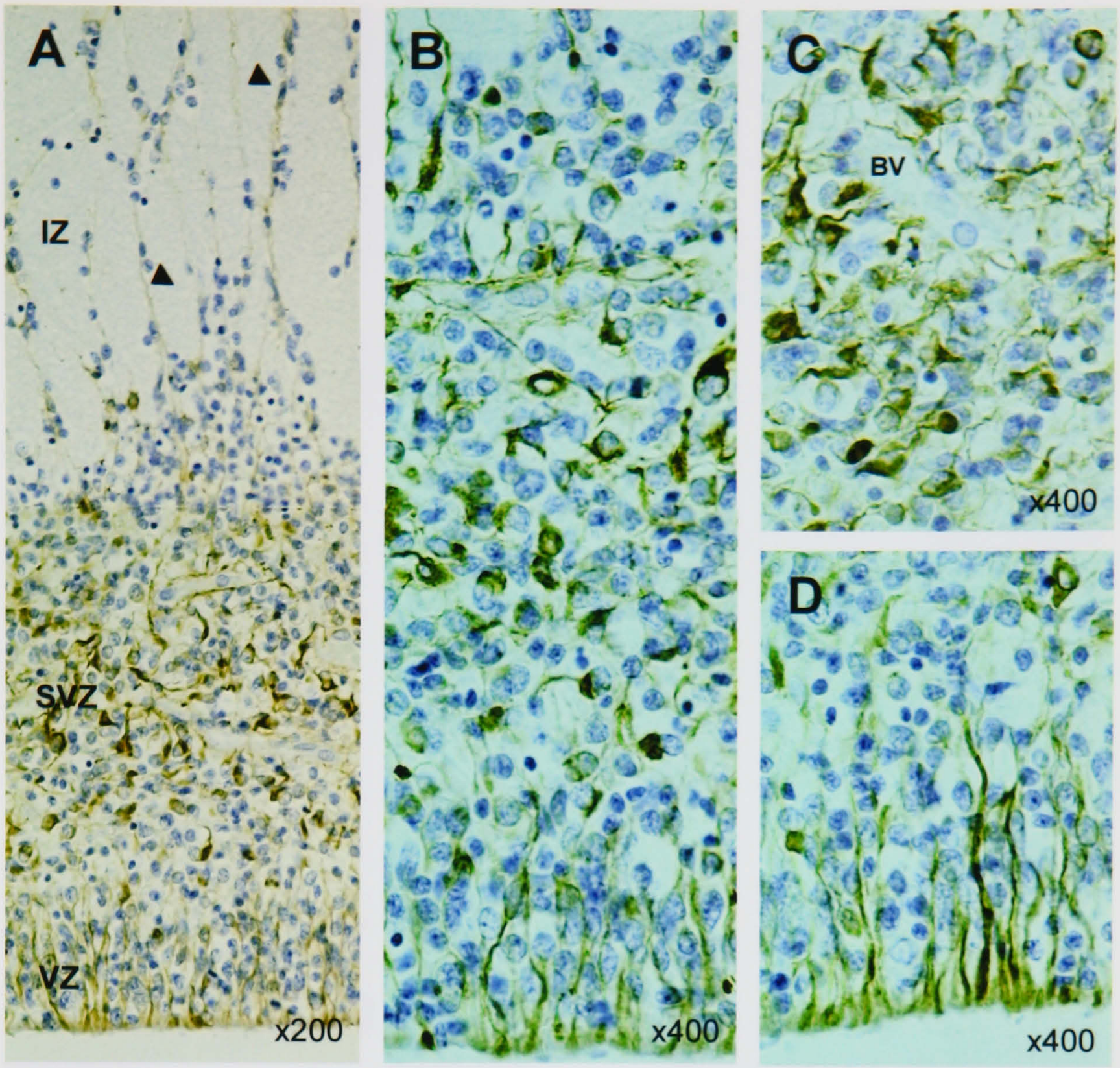


FIGURE 26

Expression of GFAP in the normal human telencephalon at 19GW

GFAP was expressed by tanycytes lining the ventricles (**A,B,D)**, and by progenitors within the germinal layers (VZ and SVZ) (**A-C**). Delicate radial glial fibers extending from the germinal layers (**A,arrowheads**), could also be identified within the intermediate zone (IZ). Closer inspection of the germinal layers, revealed that the majority of differentiating astrocyte progenitors that expressed GFAP were located in the vicinity of blood vessels (BV) onto which their processes terminated (C). Nuclei counterstained with haematoxylin.

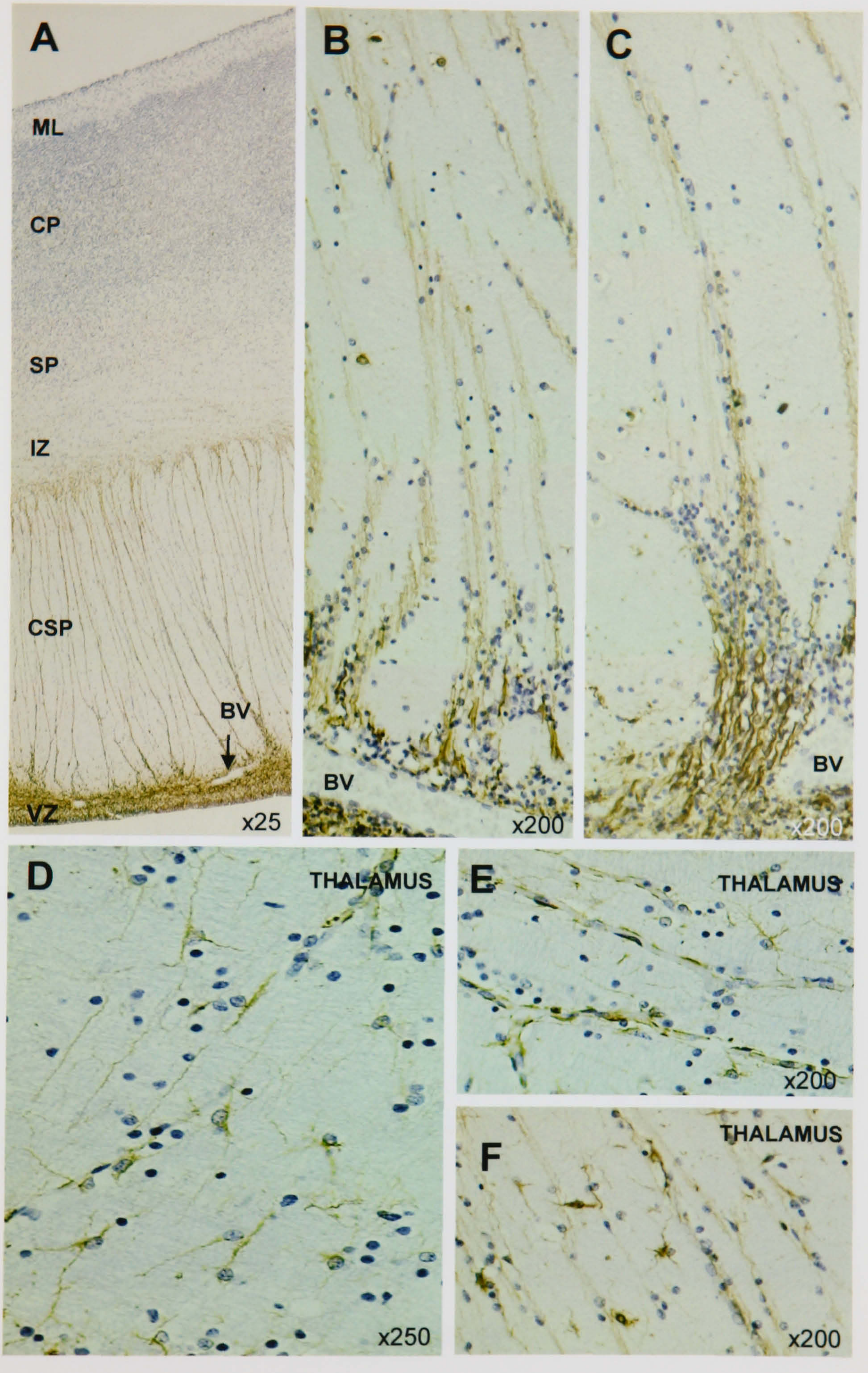


FIGURE 27

Expression of GFAP in the normal human brain, 19-20GW

Columnar arrangement of GFAP positive fibers were clearly visible within the cavum septum pellucidum, spanning this transient site (**A-C**). Intense GFAP immunoreactivity was also detected within the ventricular zone of this region, which gave rise to these fibers, and large-calibre vessels (BV) were found embedded within the VZ. At this time, GFAP positive cells in the thalamus took on more differentiated morphologies (**D**), and a significant number of these cells were clearly investing blood vessels with their processes (**E,F**). Nuclei counterstained with haematoxylin.

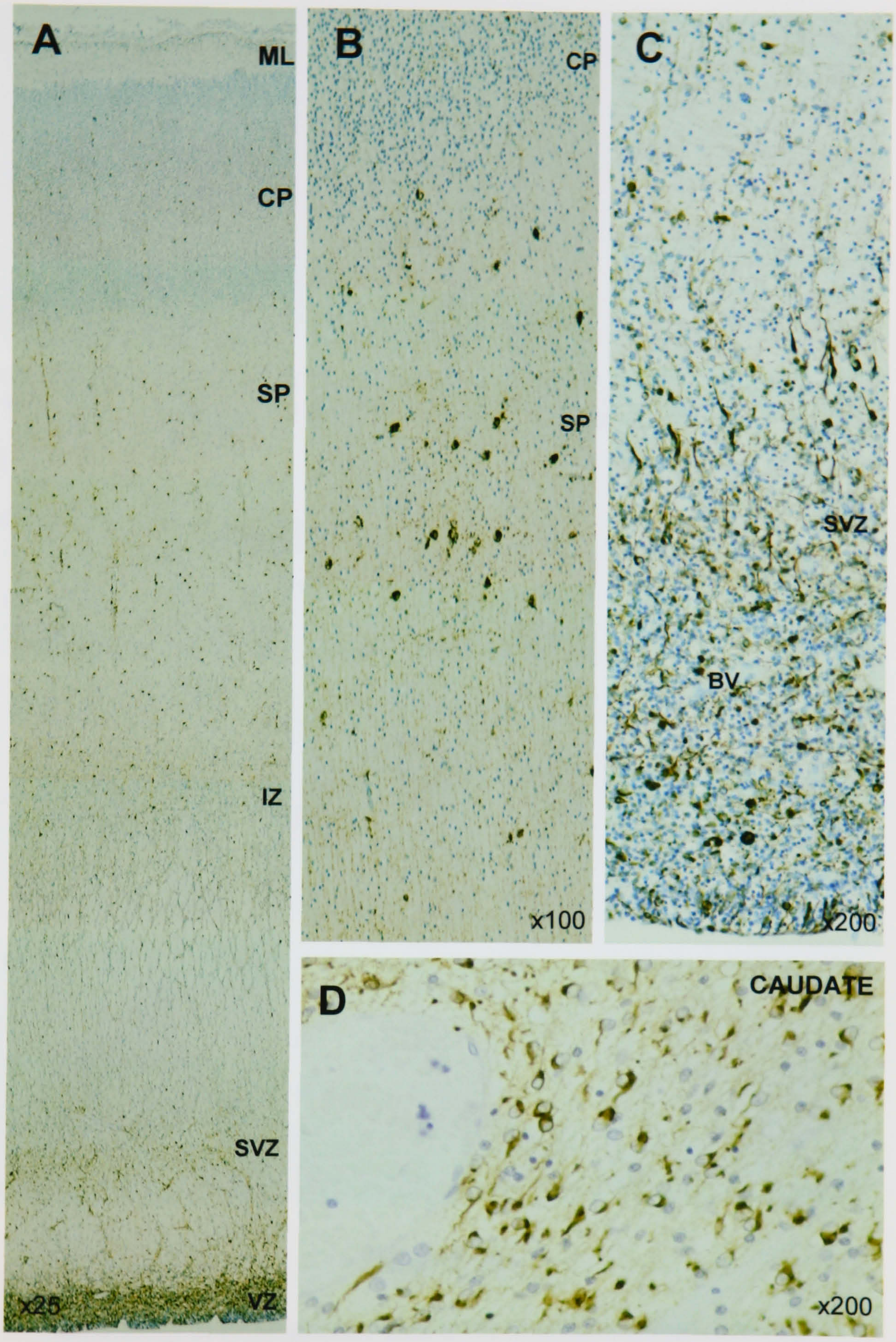


FIGURE 28

Expression of GFAP in the normal human brain, 20-21GW

There was continued expression of GFAP within the VZ and SVZ between 20-21GW (**A**). However, GFAP-positive cells could now be found interspersed throughout the intermediate zone, subplate and extending into the cortical plate (**A,B**). Focally, cells located in the subplate showed higher GFAP expression (**B**). Those within the upper aspect of the germinal layers took on a more polarised morphology, extending their processes unidirectionally toward the IZ from the SVZ (**C**), and these continued through the expanse of the cortex as thin, almost non-detectable (radial glial) fibers. In the caudate, GFAP positive astrocyte progenitors were also common (**D**). Nuclei counterstained with haematoxylin.

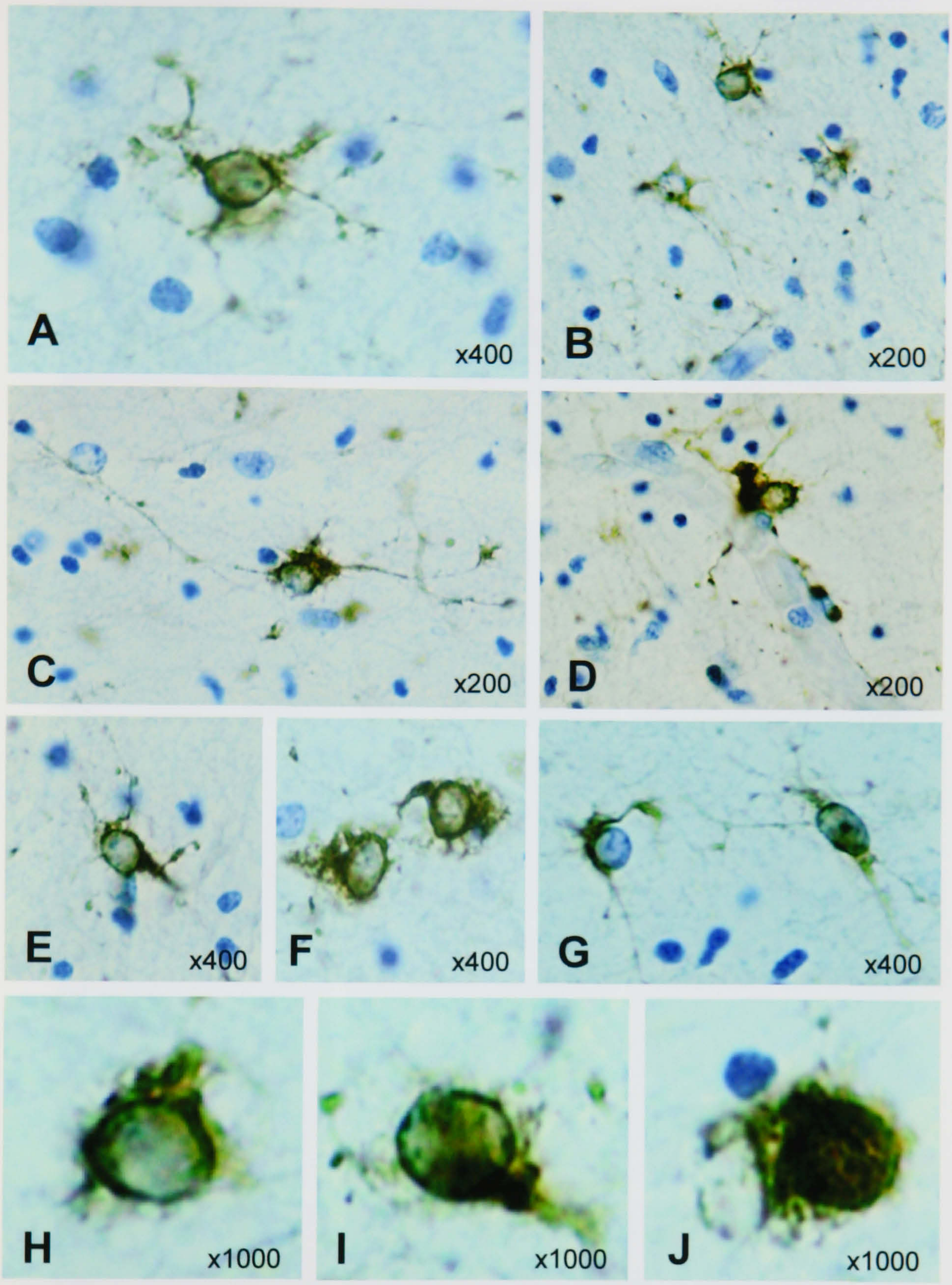


FIGURE 29

Morphology of GFAP positive cells in the intermediate zone of normal human telencephalon between 20 and 21GW

GFAP-positive astrocyte progenitors were encountered at different stages of differentiation within the intermediate zone. Isolated cells with three or more processes emitting from their cell body region (**A,B**), cells that extended delicate, thin processes in a bipolar fashion (**C**), those that were clearly associating with cortical blood vessels (**D**) as well as more immature varieties (**H-J**) were all readily identified. Nuclei counterstained with haematoxylin.

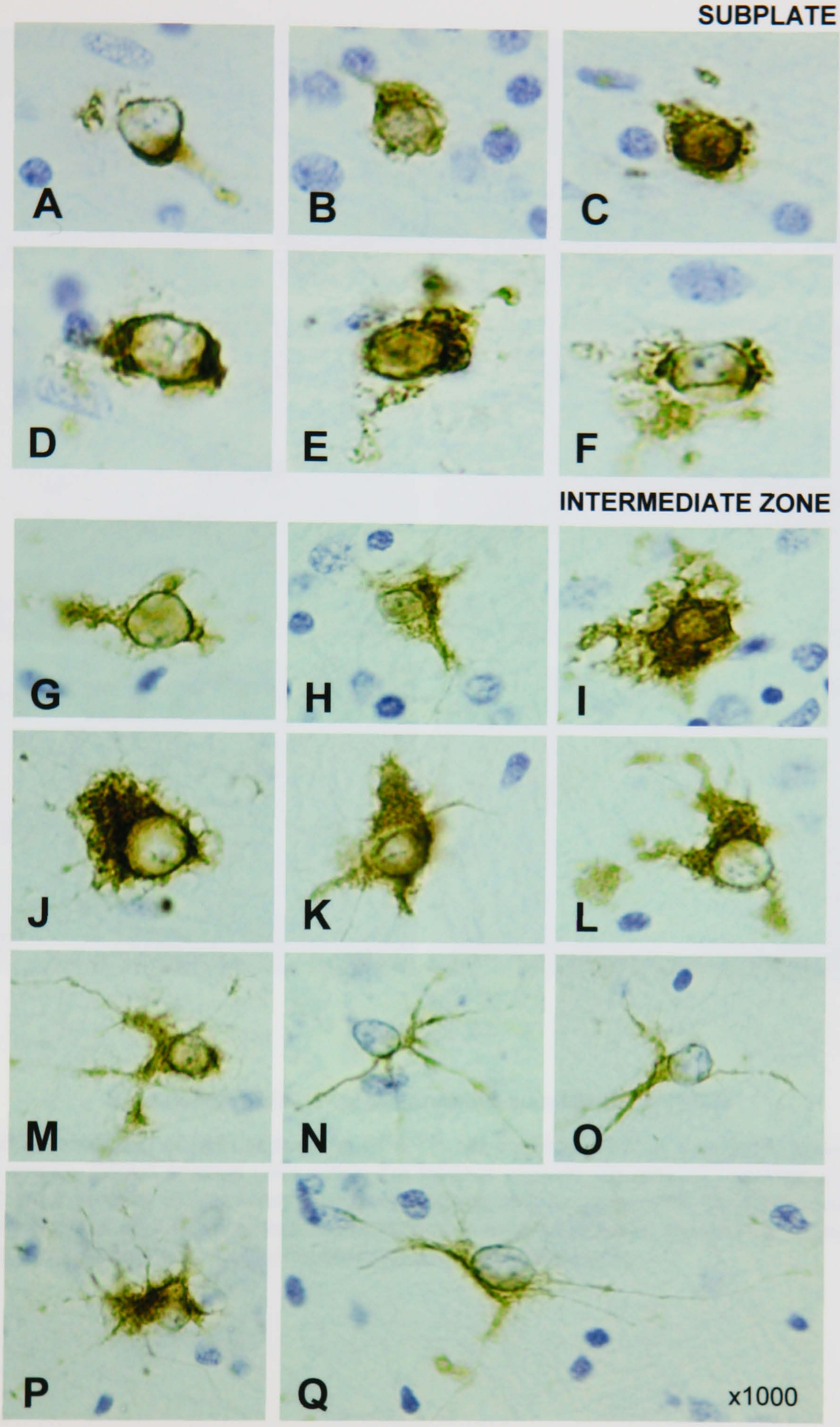


FIGURE 30

Morphology of GFAP positive cells in the subplate and intermediate zone of normal human brain at 23GW

The majority of GFAP positive cells in the subplate tended to remain in a more undifferentiated and immature morphology even at this more advanced stage in development (**A-F**). By contrast, GFAP positive cells were clearly in the process of transformation in the intermediate zone by 23 weeks (**G-Q**). There were fewer cells of the immature morphological forms (**G-L**) and higher numbers of cells possessing more mature astrocyte morphologies (**M-Q**). Nuclei counterstained with haematoxylin.

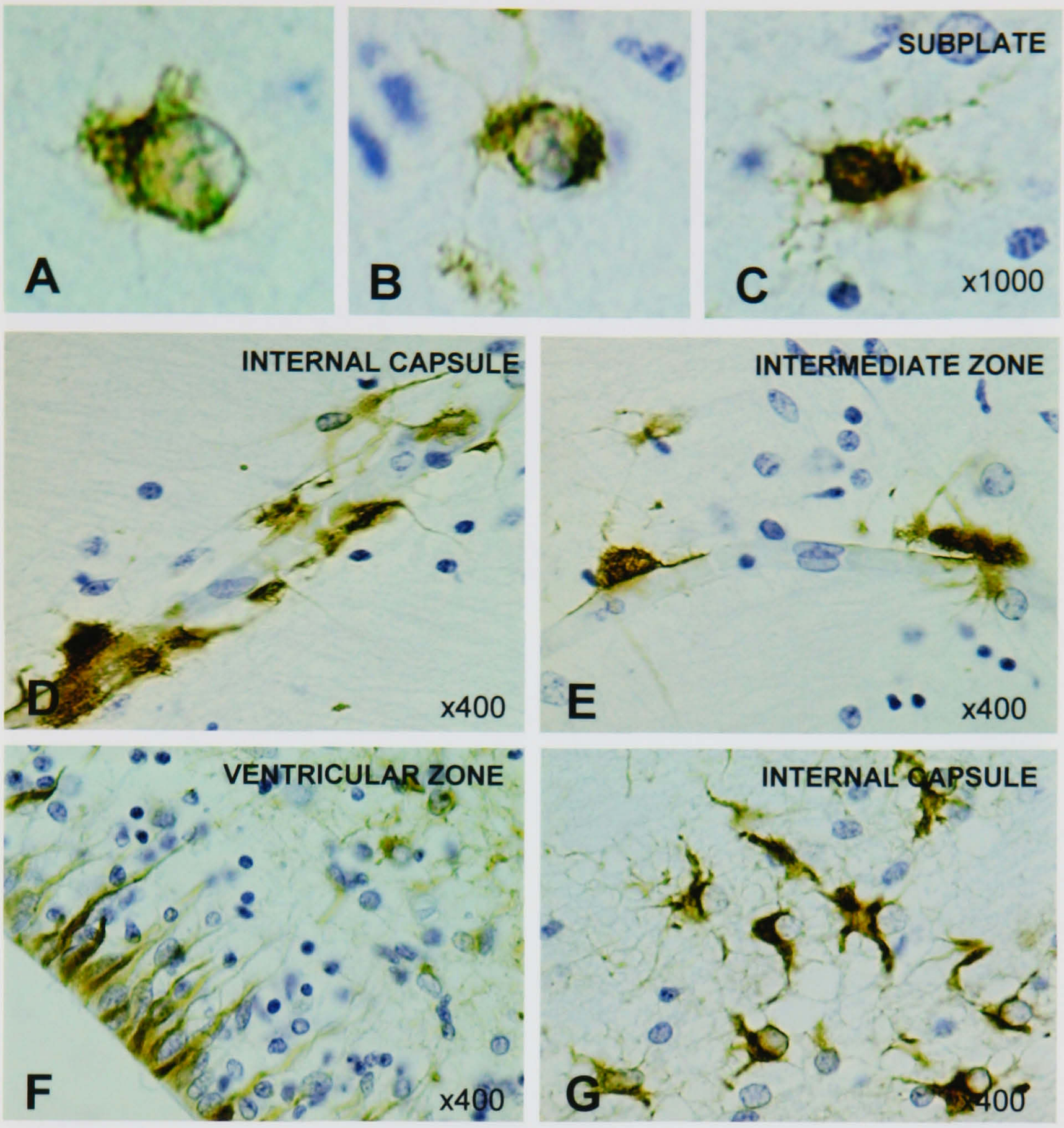


FIGURE 31

Expression of GFAP in the normal human brain at 23GW

Further examples of cells expressing GFAP in the subplate (**A-C**) of a separate case at 23 weeks. GFAP positive cells within the internal capsule (**D**) and intermediate zone (**E**) are frequently encountered clearly associating with blood vessels. Tanycytes lining the ventricles (**F**) and more asteriform immature astrocytes within the internal capsule (**G**), are also common. Nuclei counterstained with haematoxylin.

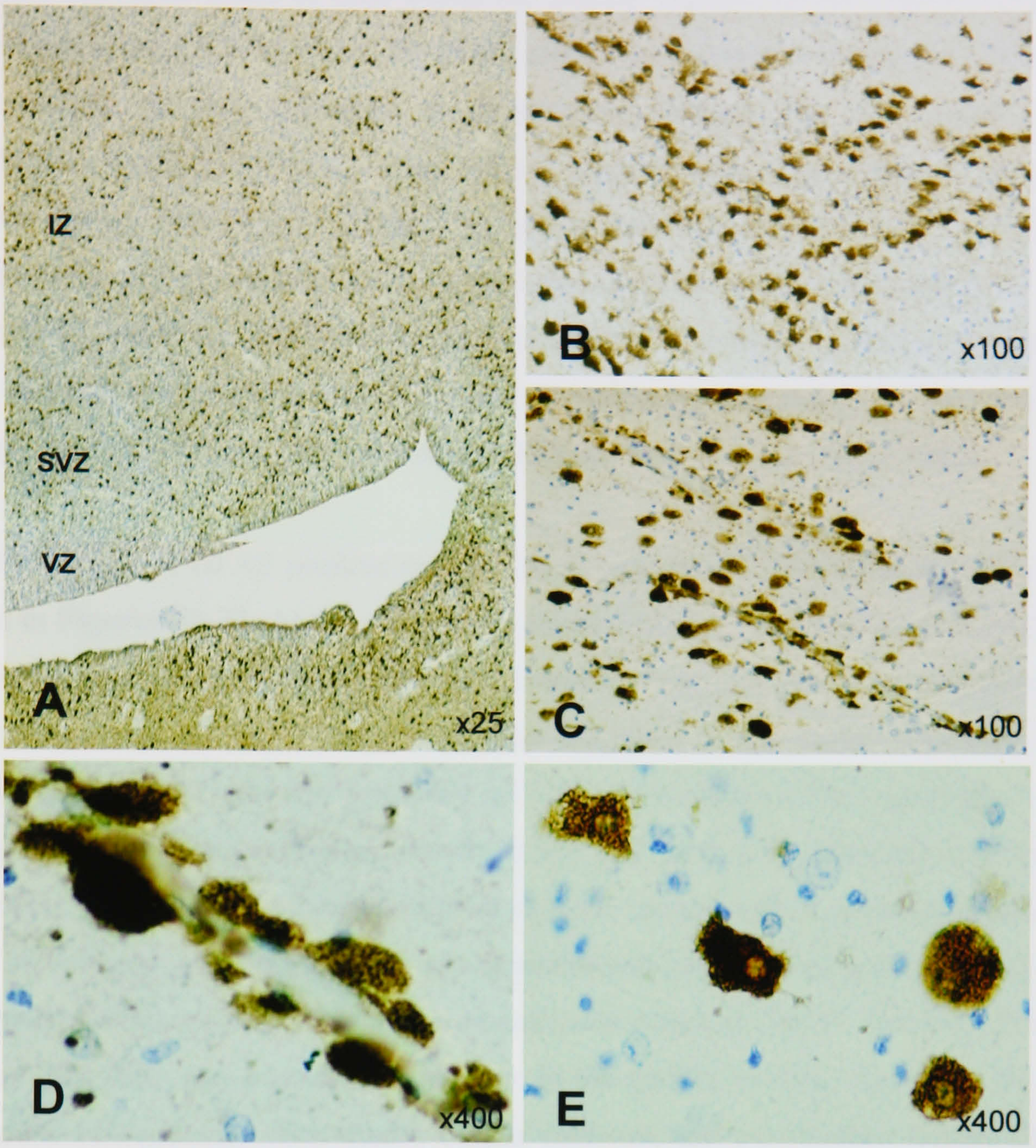


FIGURE 32

Pathological morphology of cells expressing GFAP in the human brain diagnosed with hypoxic-ischaemic injury at 26-27GW

The morphology of GFAP positive cells in foetal hypoxic-ischaemia was profoundly different from that of normal foetal control cases. Reactive changes in these cells had taken place throughout all layers of the telencephalon (**A**), and particularly on cells within white matter tracts (**B**), and in the vicinity of, or adherent to blood vessels (**C,D**). A vesicular/foamy appearance and rounded, hypertrophied morphology was the prevalent reactive change in these GFAP-expressing cells (**E**). Nuclei counterstained with haematoxylin.

Immature astrocytes could be seen within the internal capsule, GFAP positive tanycytes were still evident and regionally within the brain, astrocytes had begun to associate intimately with blood vessels, as they continued to differentiate (**Figure 31**). By comparison. in the pathological hypoxic-ischaemic state, GFAP positive cells were found to adopt marked reactive changes both in their morphology and in their intense reactivity with GFAP (**Figure 32**). Reactive changes in these transformed cells could be detected throughout the brain, and particularly notable around blood vessels. The majority resembled 'gemistocytic astrocytes' in form, similar to that described in various disturbances affecting the CNS. Clearly these changes were distinct from that presented within the normal human foetal brain.

The distribution of GFAP positive cells in the human foetal brain between 19 and 23GW, is shown in **Figures 33-37**. At the earlier time points examined, between 19 and 20GW (**Figure 33**, **Figure 34**), GFAP positive cells were found in small numbers dispersed throughout the intermediate zone. By 20GW, these cells were frequently found closely associated with blood vessels, extending several fine processes onto the endothelium. GFAP could also be detected in tanycytes lining the ventricles, focally at the pial surface (particularly covering midline structures), and on cells within the internal capsule, cavum septum pellucidum (where GFAP could be detected in linear columnar arrangements and parallel fibres), the thalamus, but most abundantly within the ventricular and subventricular zones by 20GW. Between 20 and 21GW (**Figure 35**), there was a noticeable increase in the density of cells expressing GFAP within ventricular and subventricular zones, corpus callosum and intermediate zones. There was a concomittant increase in the number of GFAP positive astrocytic fibers investing radiating blood vessels within the telencephalon. The internal capsule and claustrum were major foci for these cells. At 23GW (**Figure 36**, **Figure 37**), there were appreciably higher densities of these cells within the intermediate zone. The ventricular and subventricular zones still maintained a substantial population of cells newly expressing GFAP. The most striking distribution however, was that observed at boundaries between the putamen and globus pallidus, at the border between the caudate and the germinal matrix, surrounding the caudate, and within the internal capsule. GFAP positive cells were distinct within dorsal projections of the corpus callosum, and blood vessels in the dorsal aspect of the telencephalon (fronto-parietal regions) were considerably more heavily invested with GFAP positive cells and their processes. The fact that differentiating or recently differentiated GFAP-positive astrocytes co-localised within the same regions of the brain as microglia, was immediately identifiable. Their distribution at boundaries between nucleated areas, where microglia likewise tend to aggregate, albeit at an earlier stage in development (this phenomenon is already detectable for microglia at 17GW, rather than 23GW for astrocytes) is particularly significant.

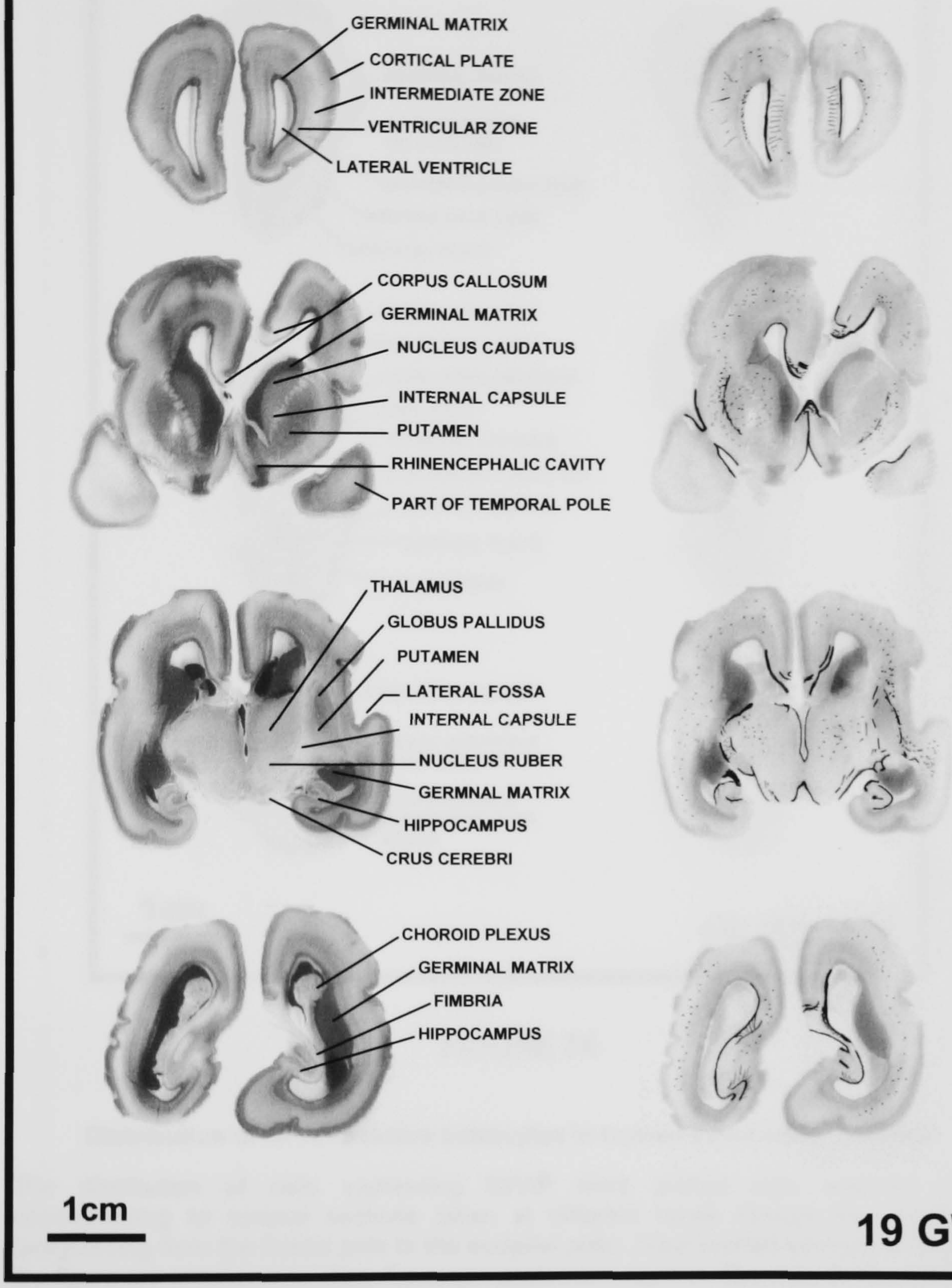


FIGURE 33

Distribution of GFAP positive astrocytes in human foetal brain at 19GW

The distribution of cells expressing GFAP were plotted onto scanned images corresponding to coronal sections taken at different levels through the foetal brain (progressing from the frontal pole to the occipital pole). Nissl-stained sections to the left of the figure are annotated to show the major anatomical regions. The plots to the right of the figure represent data gathered from three consecutive sections at each level. Both hemispheres are indicated. Solid lines represent dense astrocytic investment at the pial and ependymal (tanycytes) surfaces. Note that these are discontinuous (i.e. they do not extend continually around the surface of the brain, or the ependyma lining the ventricles). Areas where GFAP-immunoreactive astrocytes are distributed are indicated by dots. Dashed and short solid lines indicate GFAP-positive astrocytes associated with cerebral blood vessels.

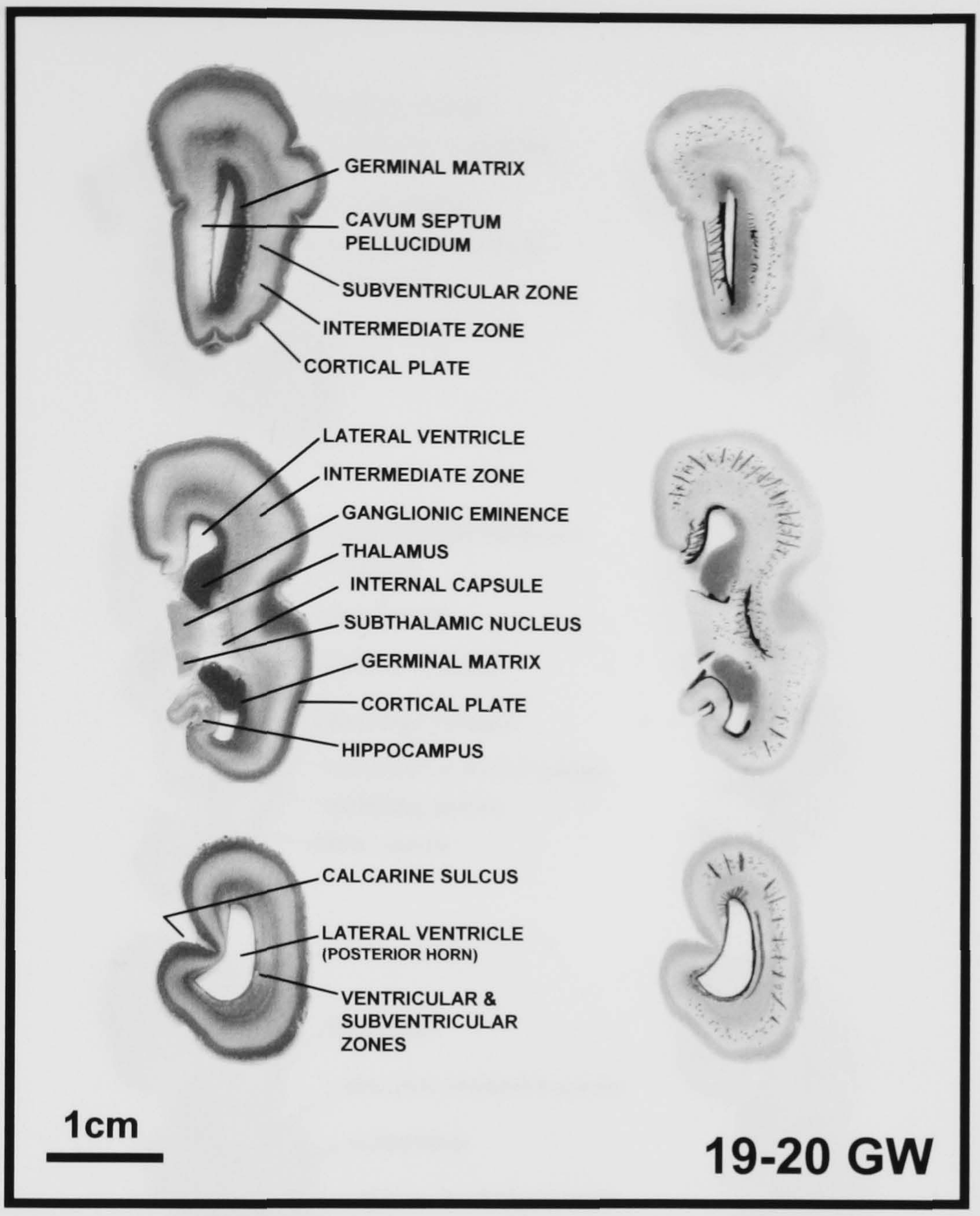


FIGURE 34

Distribution of GFAP positive astrocytes in human foetal brain 19-20GW

The distribution of cells expressing GFAP were plotted onto scanned images corresponding to coronal sections taken at different levels through the foetal brain (progressing from the frontal pole to the occipital pole). Nissl-stained sections to the left of the figure are annotated to show the major anatomical regions. The plots to the right of the figure represent data gathered from three consecutive sections at each level. One hemisphere is indicated. Solid lines represent dense astrocytic investment at the pial and ependymal surfaces or within the subventricular zone. Areas where GFAP-immunoreactive astrocytes are distributed are indicated by dots. Dashed and short solid lines indicate GFAP-positive astrocytes associated with cerebral blood vessels. A similar scheme has been adopted for Figures 35-37.

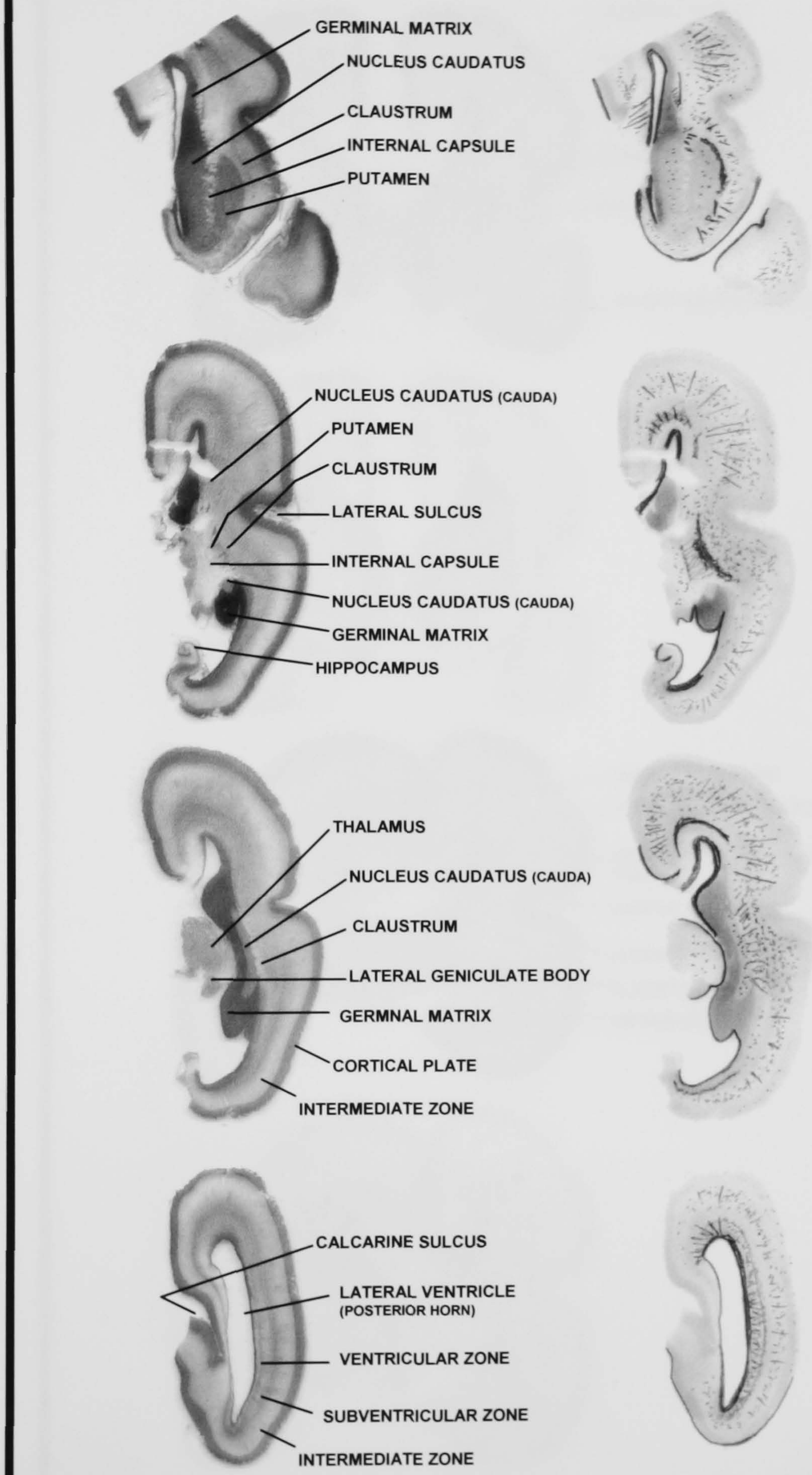


FIGURE 35

Distribution of GFAP positive astrocytes in human foetal brain 20-21GW

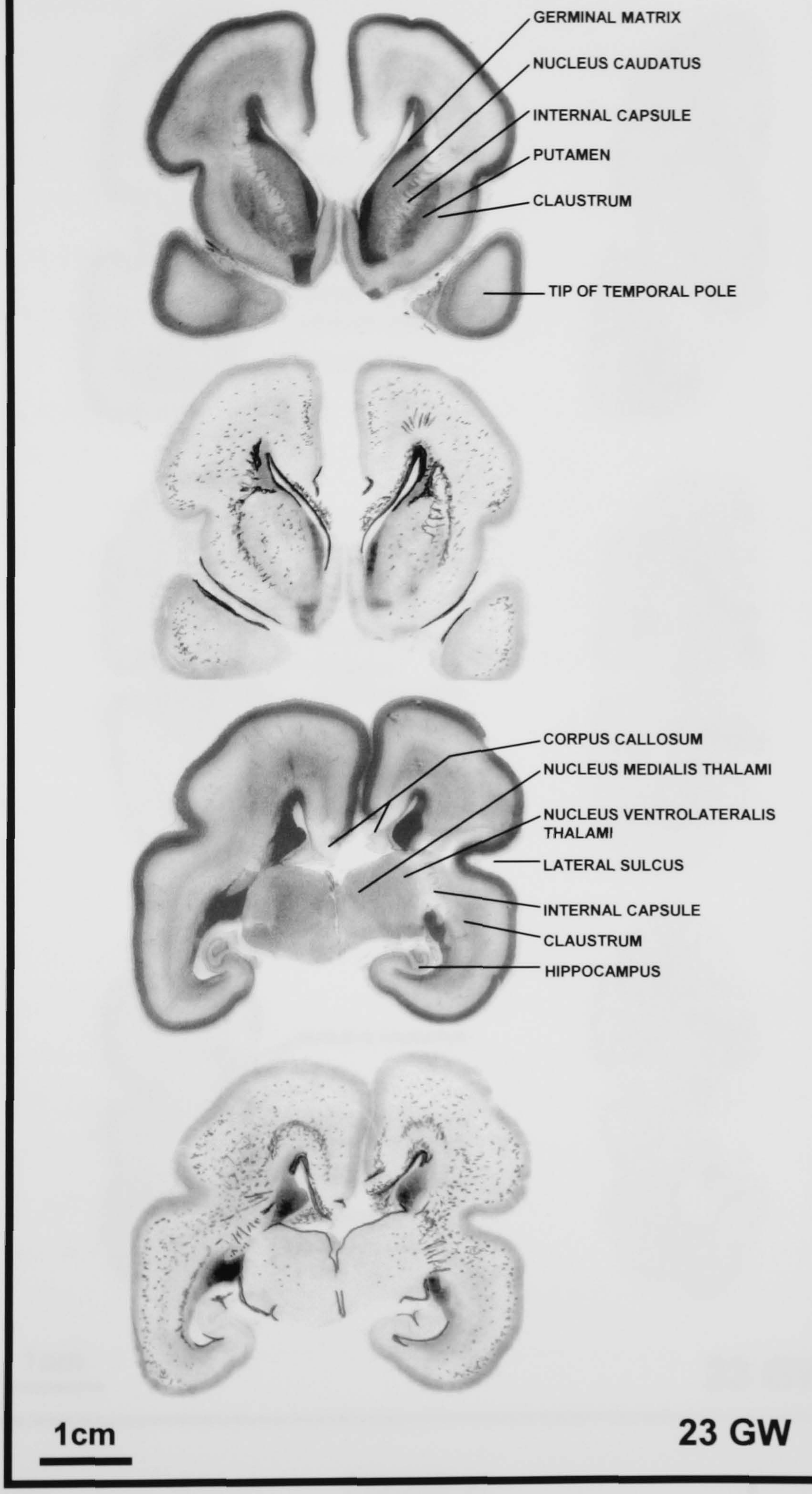


FIGURE 36

Distribution of GFAP positive astrocytes in human foetal brain 23GW (case 1)

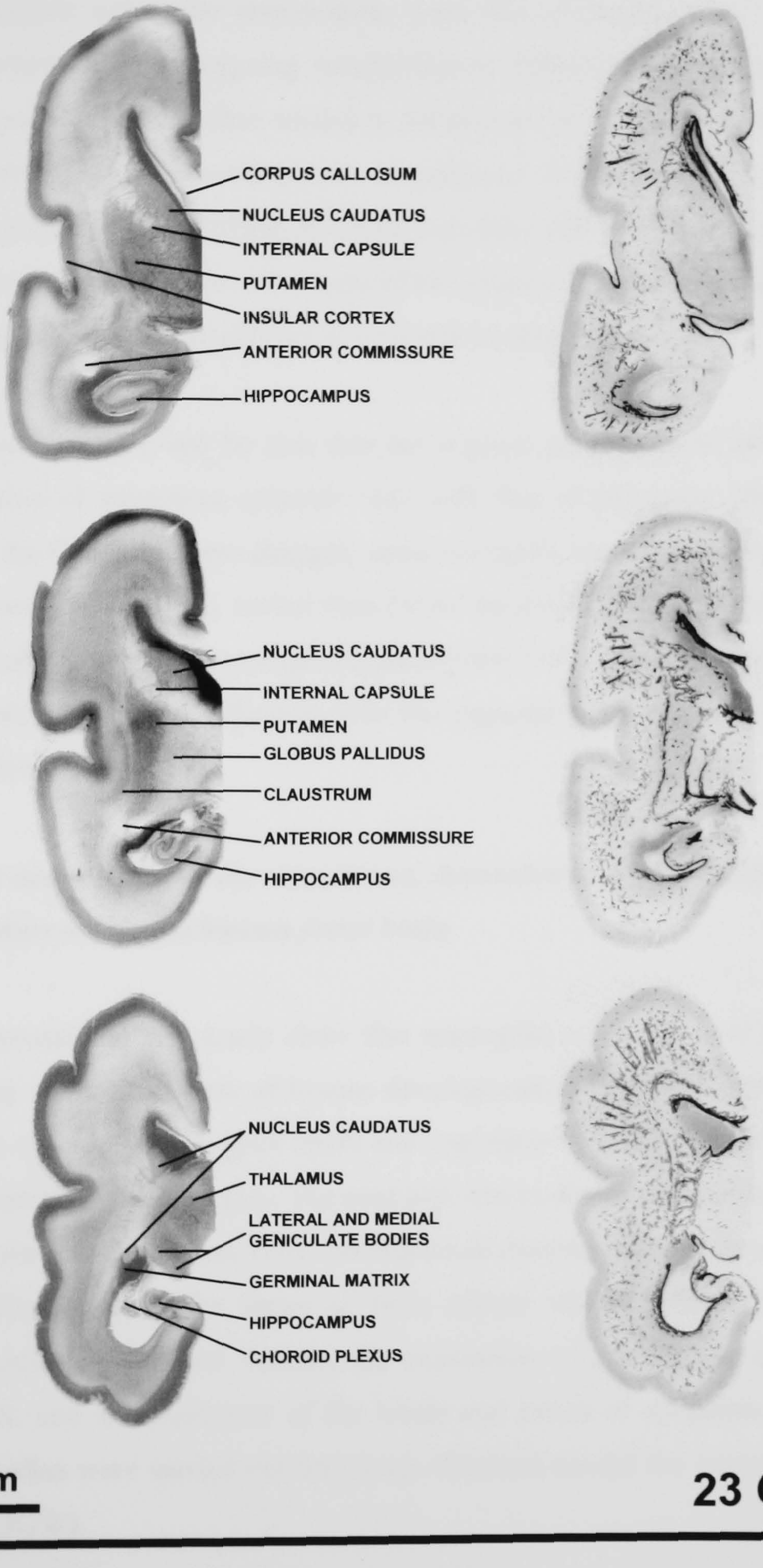


FIGURE 37

Distribution of GFAP positive astrocytes in human foetal brain 23GW (case 2)

Similarly, the distribution of GFAP-positive cells within the internal capsule, ventricular and subventricular zones corresponds closely with that of microglia in the same regions. Moreover, astrocytes within the intermediate zone, like microglia, were typically found as isolated cells which were undergoing morphological differentiation during this period. At 23GW, GFAP positive cells further tended to accumulate in the subplate laterally within the telencephalon (frontal and temporal poles), as indicated on **Figure 36**. The observation that certain blood vessels are preferentially invested with these differentiating astrocytes may attest to a differential gradient for the development of blood-brain-barrier properties, that takes place regionally within the human foetal brain at second trimester.

From these observations, it can be seen that the regional prevalence of astrocyte progenitors and differentiation of astrocytes coincide well with that of microglia. However, there is a discrepancy in the timing of these changes, since microglia appear to differentiate (within the intermediate zone and subplate), earlier than GFAP positive astrocytes. This most interesting finding will require additional investigation, particularly with respect to whether microglia can themselves exert more direct influence over the regional differentiation of astrocytes with which they co-localise.

IV. Signals associated with the recruitment, dispersion and differentiation of microglial progenitors within the human foetal brain

The studies presented so far clearly show that microglial colonisation of the CNS is taking place within the second trimester of human development, and their distribution corresponds regionally with vascularisation, glial fibers and correlates well both spatially and temporally with the differentiation of astrocytes. The next aim was to decipher possible signals that could be responsible for recruiting these cells and that could determine their regional prevalence and patterns of differentiation. The focus of these efforts was directed at the expression of adhesion molecules by cerebral vessels, the expression of chemokines and their receptors within the CNS, and an assessment of the levels and extent of apoptosis in the developing brain. These studies were carried out on tissues obtained toward the latter half of the second trimester (16-23GW).

<u>Vascular adhesion molecules</u>

Sections of the human foetal CNS were screened for expression of CD31 (PECAM-1), CD54 (ICAM-1), CD102 (ICAM-2), CD106 (VCAM-1), E- and P-selectin. Earlier, it was noted that cerebral vessels invariably expressed laminin throughout the second trimester. Furthermore, microglial 'fountains' within the germinal layers co-localised with capillaries in these regions

(**Figure 38**). The majority of cerebral vessels were also found to express PECAM-1, and expression of this adhesion molecule was particularly found on radiating cortical vessels and within capillaries in the germinal layers throughout this period (**Figure 38**).

The corpus callosum at 16GW and intermediate zone at 22GW are presented in **Figure 39** for comparing expression of adhesion molecules. In contrast to PECAM-1, ICAM-1 displayed minimal, weak and patchy staining, and VCAM-1 could not be detected (not shown). There was no change in the staining intensity or distribution of PECAM or ICAM-1 between 16 and 22GW. In contrast, ICAM-2 appeared to stain vessels more specifically and particularly within the corpus callosum at 16GW. Vessels stained with ICAM-2 were more widely distributed throughout the cortex at 22GW. Dual-labelling with markers against macrophages showed microglia to be more closely associated with ICAM-2-labelled blood vessels in the corpus callosum and on radiating cortical vessels at 22GW **Figure 39**.

Although E-selectin was absent on vessels in the telencephalon, a scant number of foetal cerebral vessels were labelled with CD62-P (P-selectin) **Figure 40**. Curiously, a few non-endothelial cells (numbering no more than nine per section) were also stained with P-selectin. These cells co-expressed CD68 (**Figure 40**), particularly on their processes, whereas CD62-P was more or less confined to the cell body. Most of these cells were of rounded morphology, and on occasion, more amoeboid cells could also be identified. They were not associated with or adjacent to blood vessels, but appeared randomly distributed throughout foetal cortical sections.

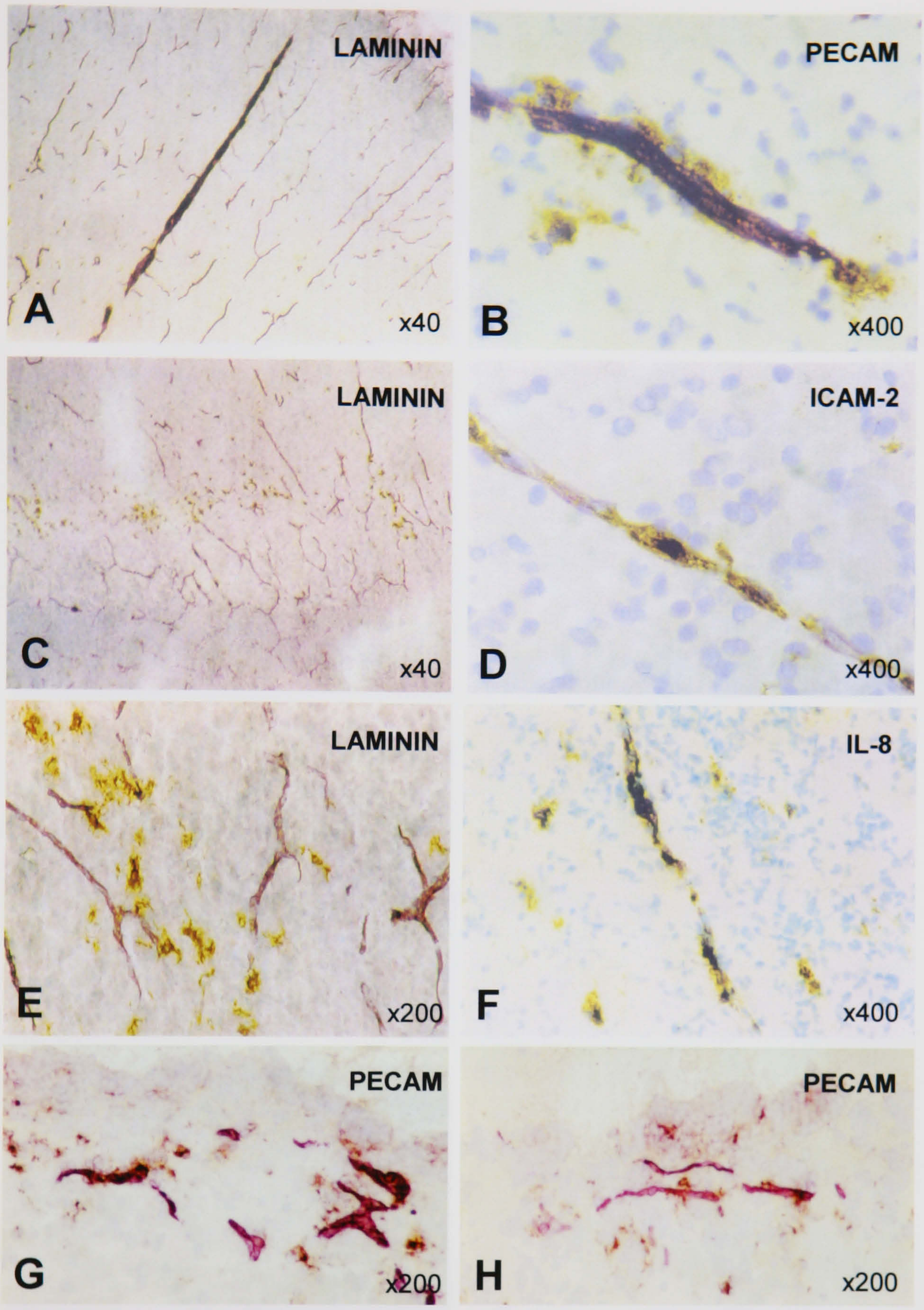


FIGURE 38

Expression of adhesion molecules in human foetal cerebral cortex, 16-22GW

(**A,C,E**) Blood vessels invariably expressed laminin throughout the second trimester. Therefore, laminin clearly defined the vascular arrangement of the human foetal brain. (**G-H**) Microglial 'fountains' wthin the germinal layer were found to co-localise with capillaries in this region. (A) laminin (violet) on cerebral vessels, 19GW; (B) PECAM-1 (violet) on vessels, CD45 (brown) identifies microglia; (C) Laminin (violet) expression on vessels within the ventricular and subventricular zones, CD68 identifies foetal microglia at 19GW; (D) ICAM-2 on a radiating cortical vessels, CD45 (brown) identifies associated mononuclear phagocytes at 22GW; (E) Higher power micrograph of (C) to show microglia (brown) and laminin (violet) in the subventricular zone at 19GW; (F) IL-8 (violet) on a cortical vessel, CD45 (brown) on microglia at 22GW; (G,H) PECAM-1 (violet) on capillaries in the germinal layer, CD68 (brown) identifies microglial 'fountains'.

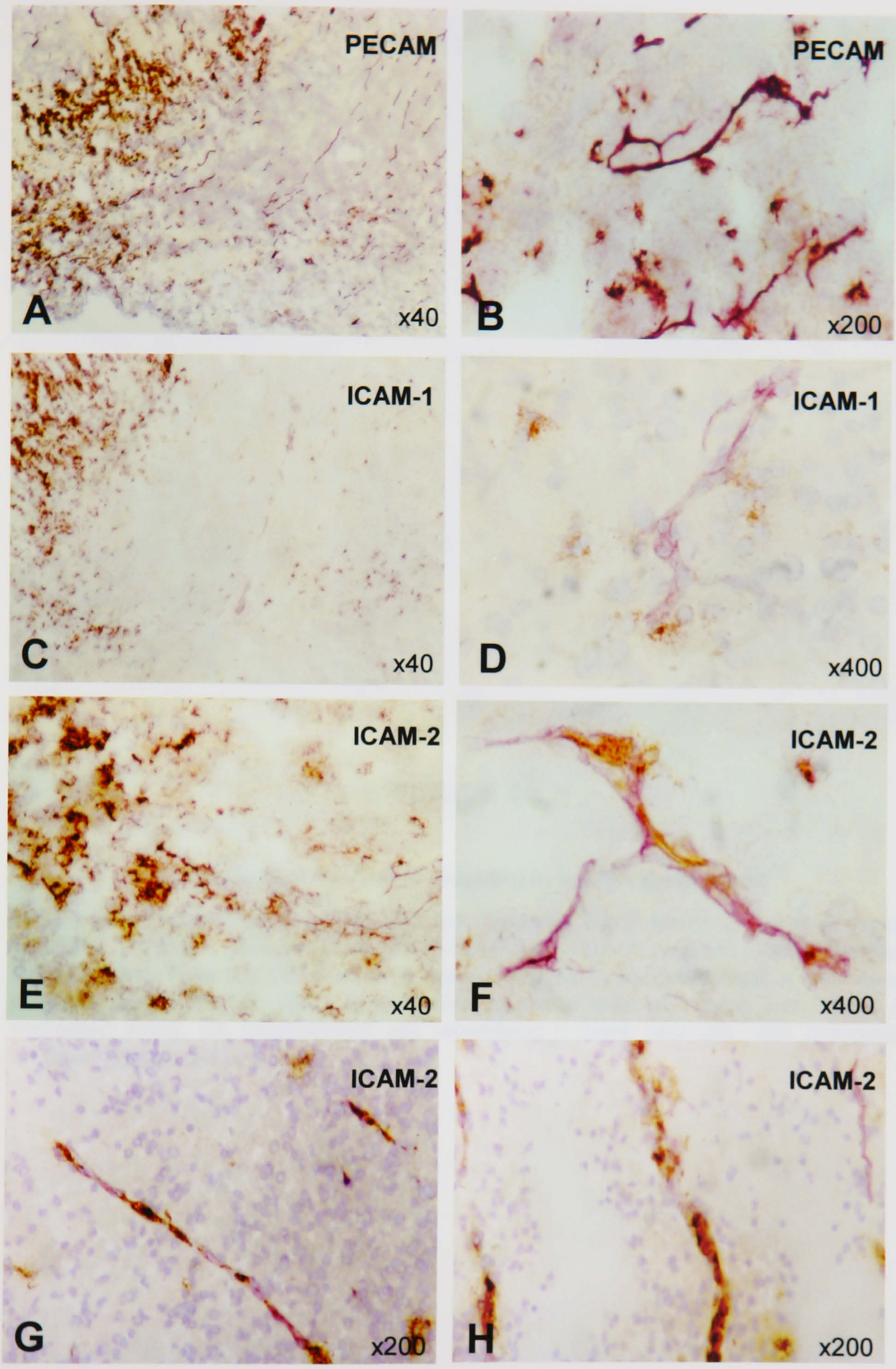


FIGURE 39

Expression of adhesion molecules in the telencephalon, 16-22GW

Double immunolabelling to demonstrate microglia and adhesion molecules on cerebral endothelium 16-22GW. Microglia are stained brown and blood vessels violet in each case. PECAM-1 (CD31) stained the majority of cerebral vessels (**A**). By comparison, ICAM-1-immunoreactive vessels were very sparse (**C**), whereas ICAM-2 expressing vessels (**E**) were frequently associated with microglial precursors within the corpus callosum (**F**) and intermediate zone (**G,H**). A-F: Human foetal corpus callosum at 16GW; G,H: radiating vessels in IZ at 22GW; A,B: PG-M1/PECAM; C,D: CD11b/ICAM-1; E-H: LCA/ICAM-2 (microglia brown, vascular adhesion molecules, violet).

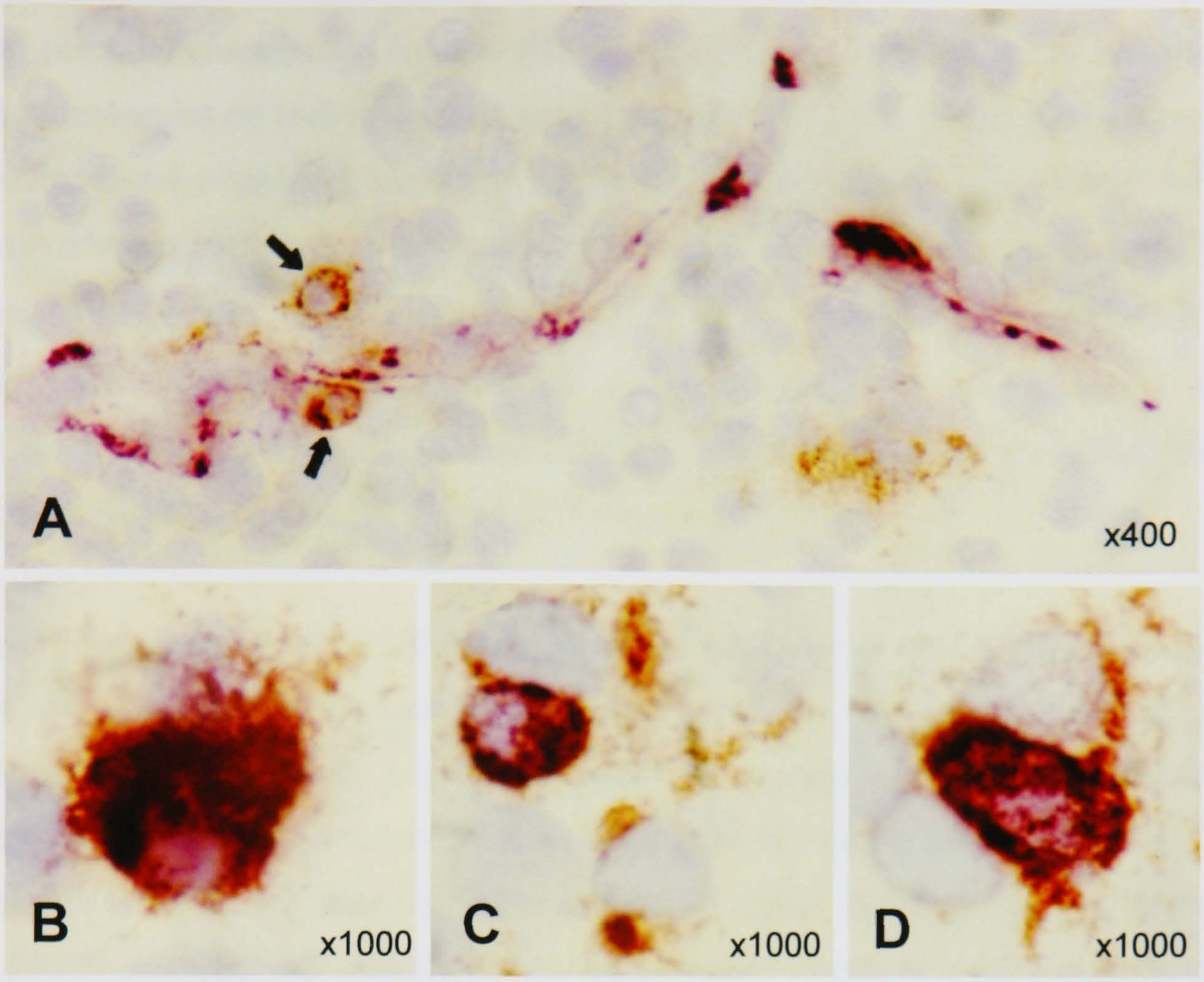


FIGURE 40

Expression of CD62-P (P-selectin) in human foetal cortex

Double immunolabelling with CD68 (PG-M1, brown)/CD62-P (violet) on cerebral cortex at 16GW. (**A**) Blood vessel stained with CD62-P and adjacent rounded CD62-P positive cells (arrows). Note the CD68 positive foetal microglia to the lower right of the vessel distinctly lacks CD62-P. (**B,C**) Dual-labelled spherical cells of varying size, found in nucleated areas. (**D**) Dual-labelled cell resembling a foetal microglia, with processes surrounding an adjacent cell.

Chemokines and chemokine receptors

Several potential chemotactic factors, including chemokines, are known to activate integrin adhesion molecules on leukocytes and macrophages, causing associated reorganisation of the cytoskeleton and triggering migration of these cells (Glabinski et al. 1996). In order to ascertain whether such signals and their receptors were expressed during the second trimester. sections of the human foetal brains (16-22GW) were screened for the β-chemokines: MIP-1α, MIP-1β, MCP-1, MCP-3, RANTES, the α-chemokines: IL-8, IP-10, SDF-1 and their receptors: CCR2, CCR5, CXCR1 and CXCR4. The expression of the CX_3C chemokine Fractalkine was also assessed.

Of the chemokines examined, MCP-1 in particular was noted to label a band of cells diffusely within the subplate and predominantly lower aspect of the cortical plate (**Figure 41**). Additional immunoreactivity was noted within the corpus callosum, the ventricular and subventricular zones and in the choroid plexus (**Figure 41**). Specifically, expression of MCP-1 localised to the subplate, was limited to the lateral aspect of the developing telencephalon from 19GW onwards, and notably absent from dorsal, ventral or medial regions. Although astrocytes are capable of producing MCP-1 in tissue culture, as shown in this study (see Chapter 4) and by others (Glabinski et al. 1996; Hayashi et al. 1995), MCP-1 expression was more widely distributed within the subplate and did not co-localise solely with with GFAP-positive cells *in situ* within the human foetal brain.

MIP-1α was not expressed appreciably within the cortical plate or sub-plate. Instead, this chemokine specifically labelled cells within the ventricular zone (not shown) and the intermediate zone (**Figure 42**). Double-labelling with an antibody to CD68 revealed that MIP-1α positive cells in the intermediate zone were foetal microglia. Notably, these cells commonly appeared in pairs, suggesting that they had recently divided. Paired cells in this zone expressed PCNA, also implying recent division (**Figure 19**). Within the ventricular zone, it was likely that both MIP-1α and MCP-1 were labelling blood vessels as well as neuronal or glial progenitors. These observations show that microglia may be responding to differential chemokine gradients of MCP-1 expression, which may account for their migration throughout the telencephalon during the mid-to-late second trimester.

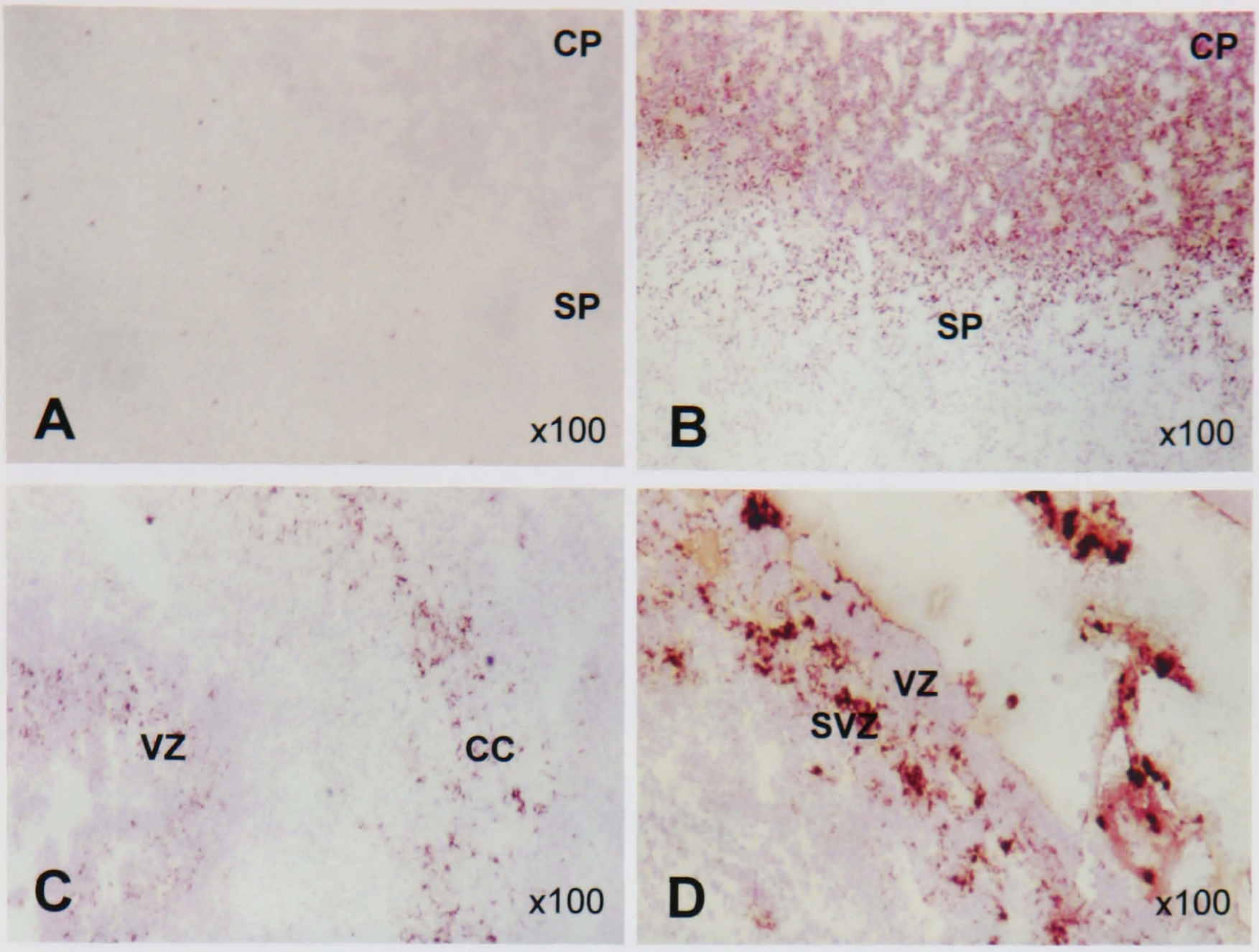


FIGURE 41

MCP-1 expression in human foetal telencephalon at 22GW

(**A**) an area of cortex lacks expression of MCP-1. (**B**) adjacent area of cortex shows expression of MCP-1 immunohistochemistry (light haematoxylin nuclear counterstain). (**C**) MCP-1 is also expressed by cells within the ventricular zone (VZ) and projections of the corpus callosum (CC). (**D**) heavily CD68 (brown) and MCP-1 (violet) immunostained cells confined to the ventricular/subventricular zones and the choroid plexus. Abbreviations: (CP) cortical plate, (SP) subplate, (VZ) ventricular zone, (SVZ) subventricular zone, (CC) corpus callosum.

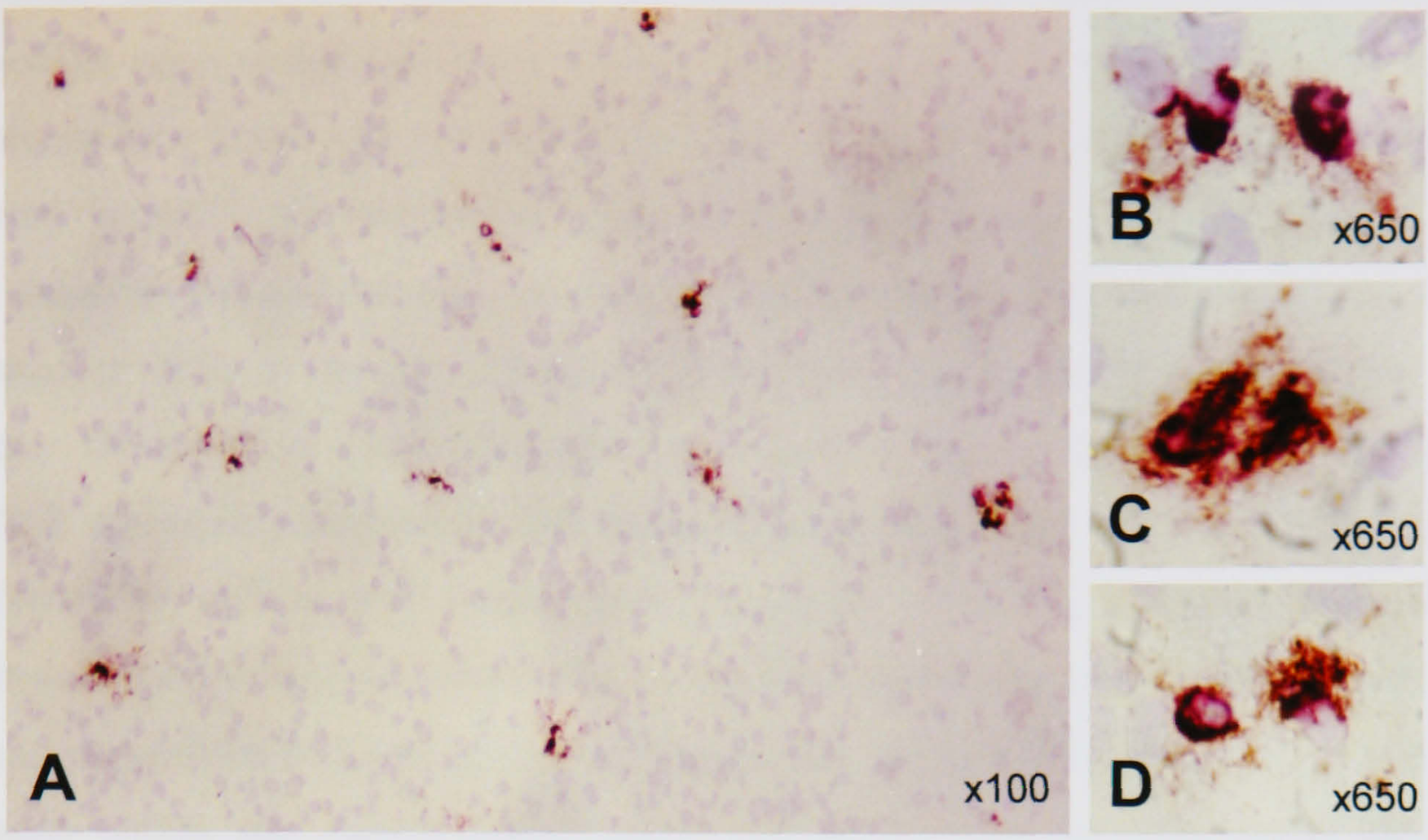


FIGURE 42

Dual immunohistochemical labelling with MIP-1α and CD68 (PG-M1) within the intermediate zone at 22GW

(**A**) Double-labelled cells within the transitory white matter (intermediate zone). The cortical plate is to the top of the figure. (**B-D**) Higher magnification of double-labelled cells within the intermediate zone. The majority of these double-stained cells appear in pairs and may have recently divided. CD68: brown, MIP-1α: violet.

Fractalkine was diffusely expressed by radiating cortical vessels passing through the intermediate zone, within the corpus callosum, and in patches on discrete cell clusters within the intermediate zone and subventricular zone from 19GW (**Figure 43**). RANTES was dispersed on cells in the intermediate zone and more specifically within the subplate and to a lesser extent in the cortical plate as early as 16GW (**Figure 44**). This chemokine was also expressed on cells in the choroid plexus. Therefore, expression of RANTES was another candidate for recruiting foetal microglia to the cortical plate and subplate. Immunoreactivity with IL-8 was mainly associated with radial blood vessels within the intermediate zone, and occasionally on cells at perivascular sites (**Figure 44**, refer also to **Figure 38F**). IL-8 was further expressed within the ventricular zone, although this chemokine was distinctly absent on some populations of foetal microglia (**Figure 44**). Neither MIP-1β, IP-10, or SDF-1 were expressed within the human foetal brain, and MCP-3 showed very weak and diffuse immunoreactivity confined to the ventricular zone and occasionally associated with tanycytes (not shown).

Dual-label immunohistochemistry revealed that foetal microglia within the intermediate zone and subplate expressed CCR2 (**Figure 45**). However, immunoreactivity with CCR2 was not confined solely to microglia and by 23GW, expression of this receptor was more widespread within the intermediate zone, subplate and cortical plate, more intense within the subplate and lower cortical plates (**Figure 46**). CCR2 was also expressed on a proportion of cells within the SVZ and corpus callosum, and intensely within the ventricular zone (**Figure 45**). CCR5 was found within the corpus callosum and subventricular zone at 22GW and on dispersed cells within the intermediate zone by 23GW (**Figure 46**). Expression of CXCR4 was intense within the same regions as CCR5 (**Figure 47A,B**), and with dual-label immunohistochemistry, identified on foetal microglia within the ventricular zone (**Figure 45E,F**). Focal expression of this chemokine receptor was also localised to the human foetal spinal cord at 9GW (**Figure 47 C,D**). Convincing expression of CXCR1 could not be determined within the human foetal nervous system using conventional immunohistochemistry.

By comparison, the chemokines MCP-1, MIP-1α and RANTES and the chemokine receptors CCR2, CCR5 and CXCR4 were all found to be associated with blood vessels in sections from the frontal cortex of a normal infant (**Figure 48** and **Figure 49**). Unlike MIP-1α, the immunoreactive expression pattern for MCP-1 and RANTES (patchy and discontinuous) may have been associated with perivascular cells that reside around blood vessels, rather than vascular endothelium *per se*. Likewise the pattern of immunoreactivity seen with CCR2, CCR5 and CXCR4 may have been associated to some extent with perivascular cells, and in the case of CCR5 were also related to cells in the vicinity of blood vessels (**Figure 49**).

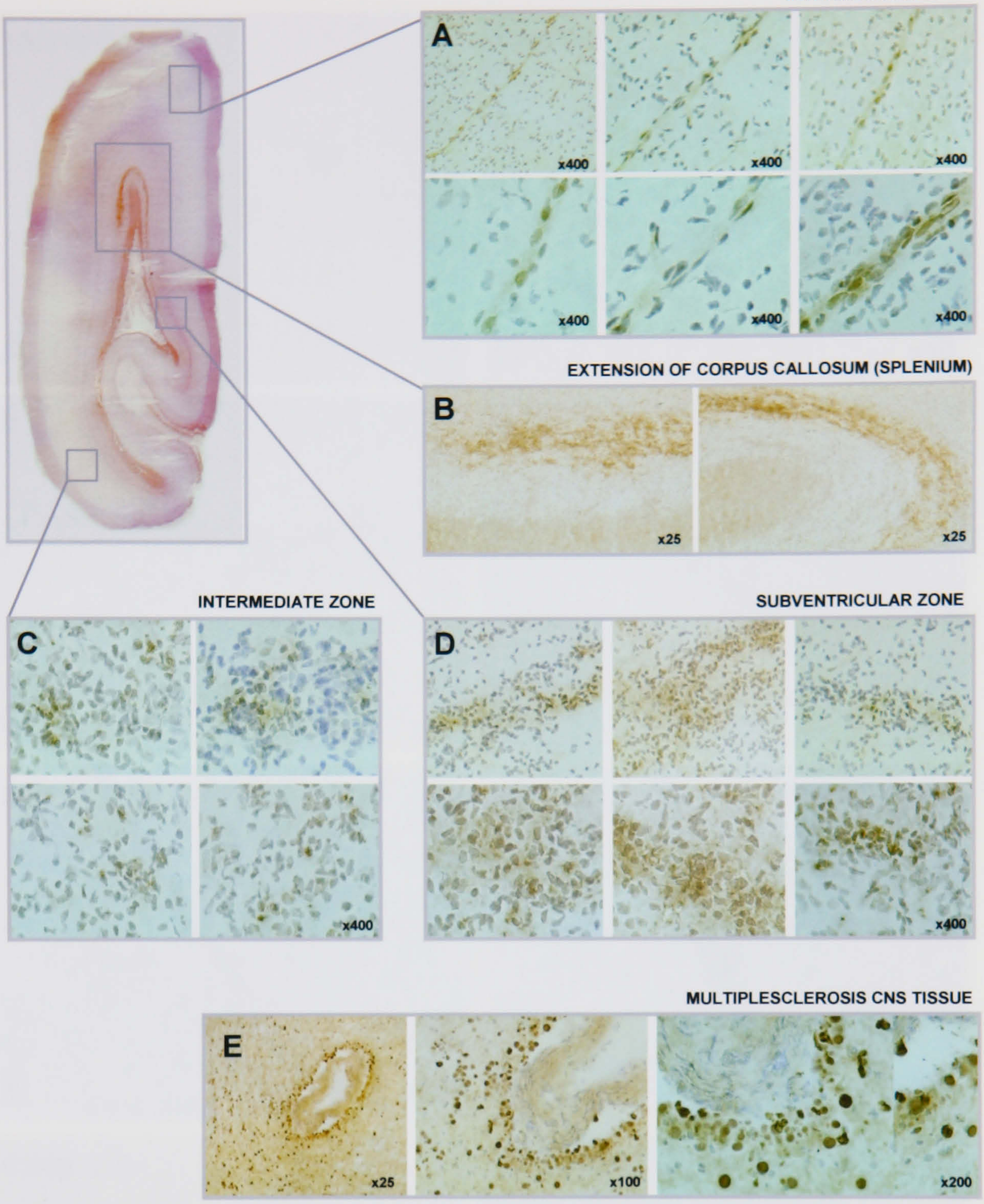


FIGURE 43

Expression of Fractalkine in human telencephalon at 19GW

Fractalkine was expressed diffusely on cerebral endothelium of radial cortical vessels (**A**) and within the ventricular zone. Strong expression was found on cells within the splenium of the corpus callosum (co-localising with microglia and astrocyte progenitors) (**B**). Expression of this chemokines was also found in patches or clusters of cells located within the intermediate zone (**C**), and the subventricular zone (**D**). In positive control tissue from a case with multiple sclerosis, fractalkine was intensely immunoreactive on perivascular infiltrating cells within the demyelinating plaque (**E**). Nuclei counterstained with methyl green.

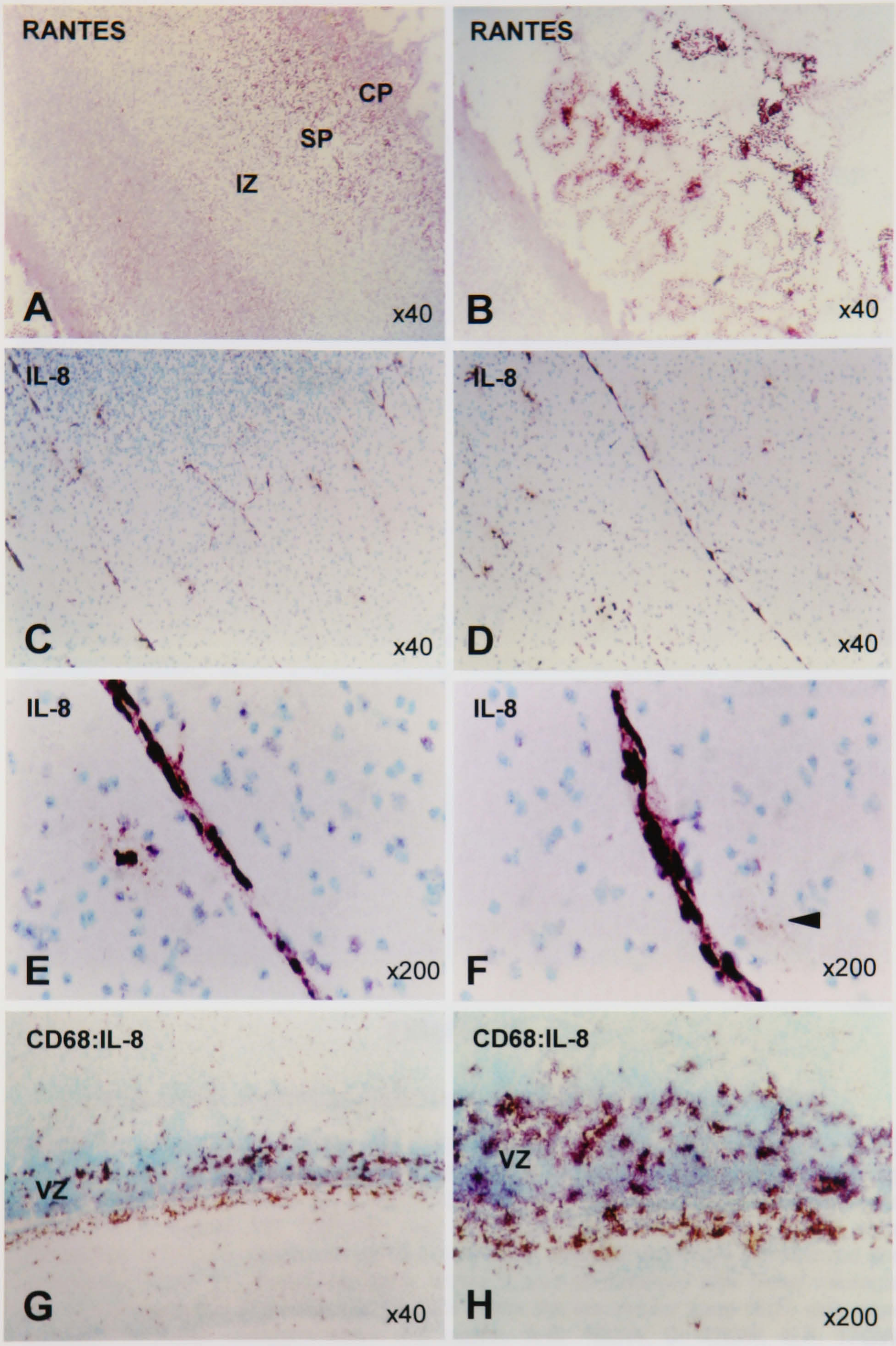


FIGURE 44

Expression of RANTES and IL-8 in human foetal telencephalon

(**A**) RANTES (violet) is expressed in the intermediate zone (IZ), subplate (SP) and to some extent within the cortical plate (CP) at 16GW. (**B**) RANTES is also widely expressed by cells within the choroid plexus. (**C-F**) IL-8 immunoreactivity is largely associated with radial blood vessels within the intermediate zone, and occasionally expressed on cells in perivascular sites (**E**), at 23GW. (**G,H**) Although IL-8 is also expressed within the ventricular zone (violet), this chemokine is not present on some populations of microglia (brown), see also (**F**) (arrowhead). A-F haematoxylin counterstain, G,H: methyl green counterstain.

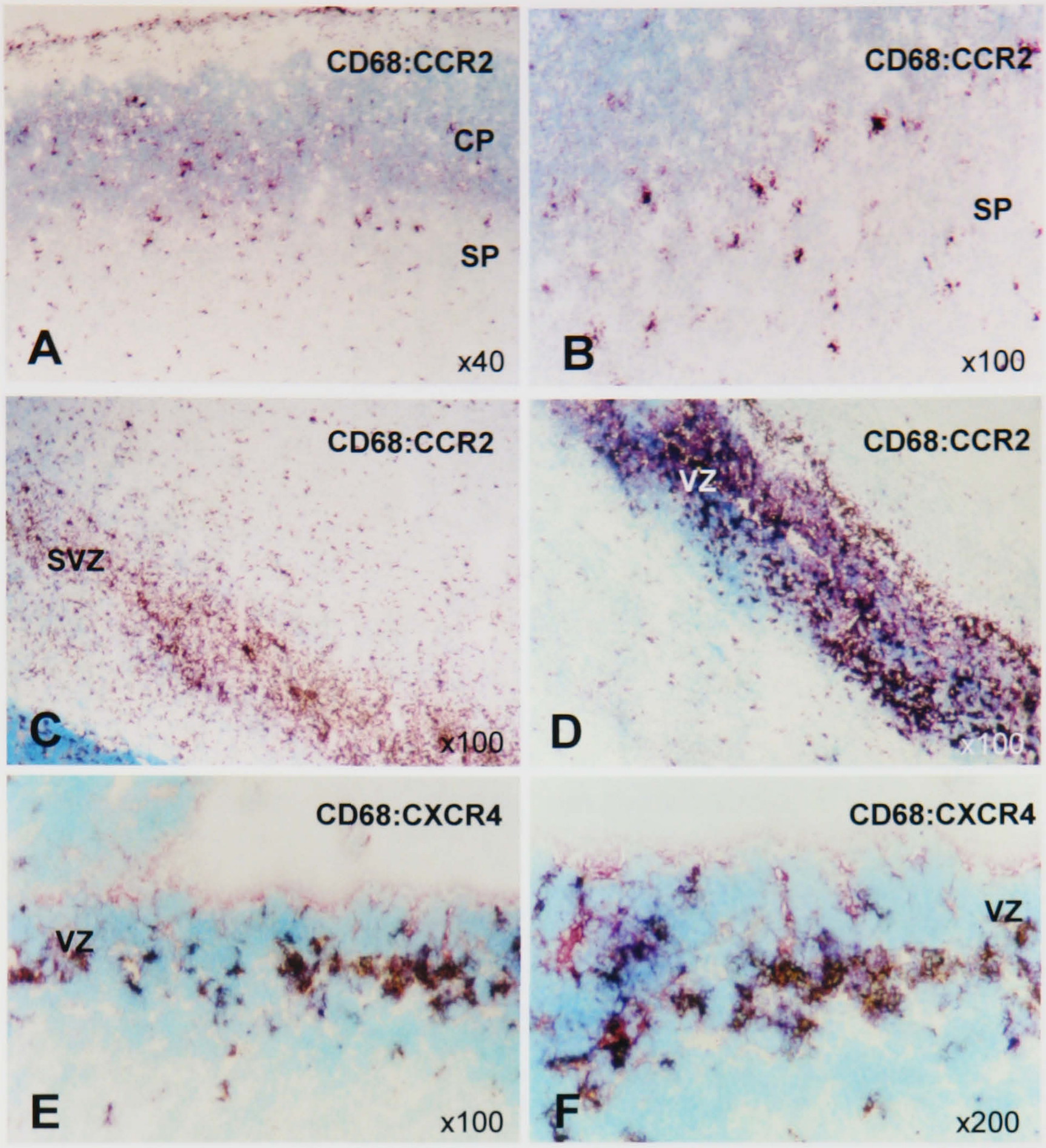


FIGURE 45

Expression of CCR2 and CXCR4 in human telencephalon, 17-22GW

Double immunolabelling of CCR2 with CD68 identified foetal microglia expressing CCR2 within the intermediate zone and subplate (**A,B**). However, not all immunoreactivity within the intermediate zone (or the cortical plate) was accounted for by microglia alone (refer also to Figure 48). Similarly, only a proportion of microglia in the SVZ were expressing CCR2 (**C**). Expression of this chemokine receptor was more pronounced and widespread within the ventricular zone, where it also co-localised with foetal microglia (**D**). By contrast, the expression of CXCR4 within the ventricular zone (**E,F**) was more restricted to foetal microglia within this region. A-C: 19GW, D: 22GW, E,F: 17GW. Microglia: brown, CCR2/CXCR4: violet. Nuclei counterstained with methyl green.

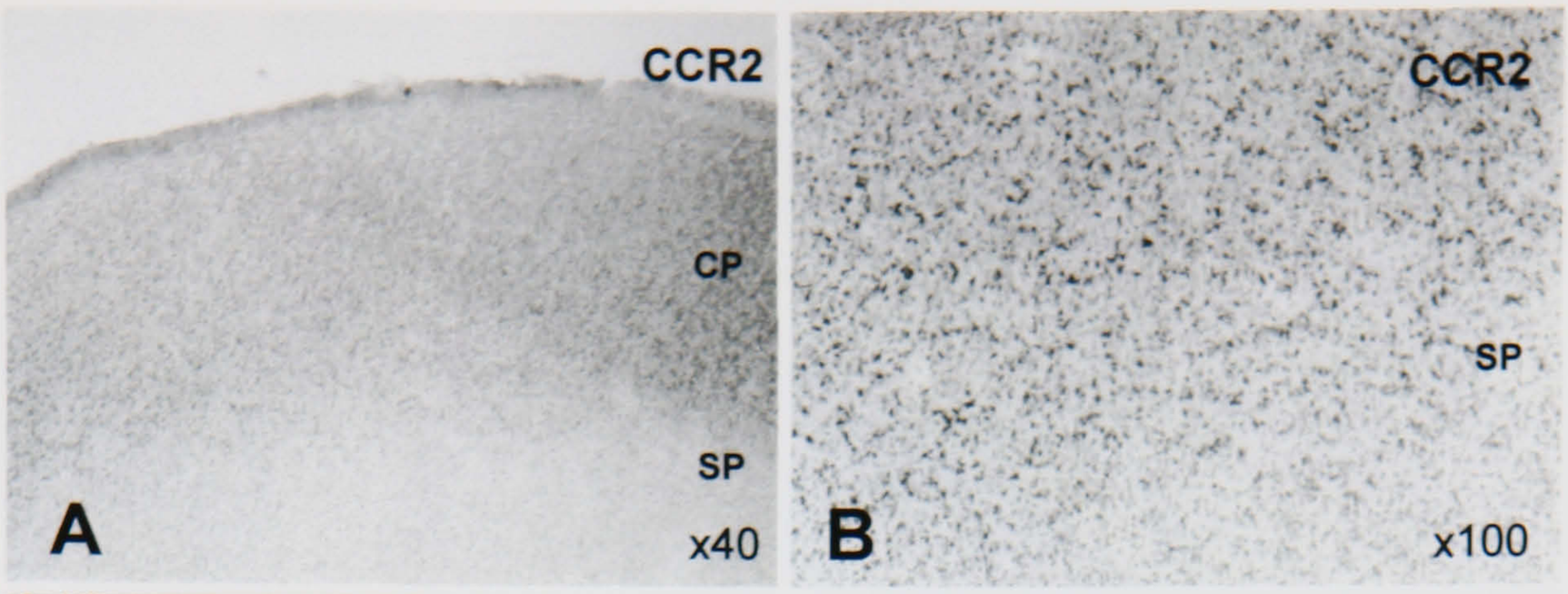


FIGURE 46

Expression of chemokine receptors in normal human telencephalon 23GW

By 23GW, the expression of CCR2 was particularly pronounced within the lower cortical plate, subplate and intermediate zones (**A-D**). Cellular profiles within the subplate and lower cortical plate were particularly distinct at this stage of development (**C**) and were more likely to be neuronal progenitors/differentiating neurons rather than microglia. Distinct cellular profiles expressing CCR5 were also dispersed within the intermediate zone of the telencephalon around this period (**E-F**). CP: cortical plate; SP: subplate.

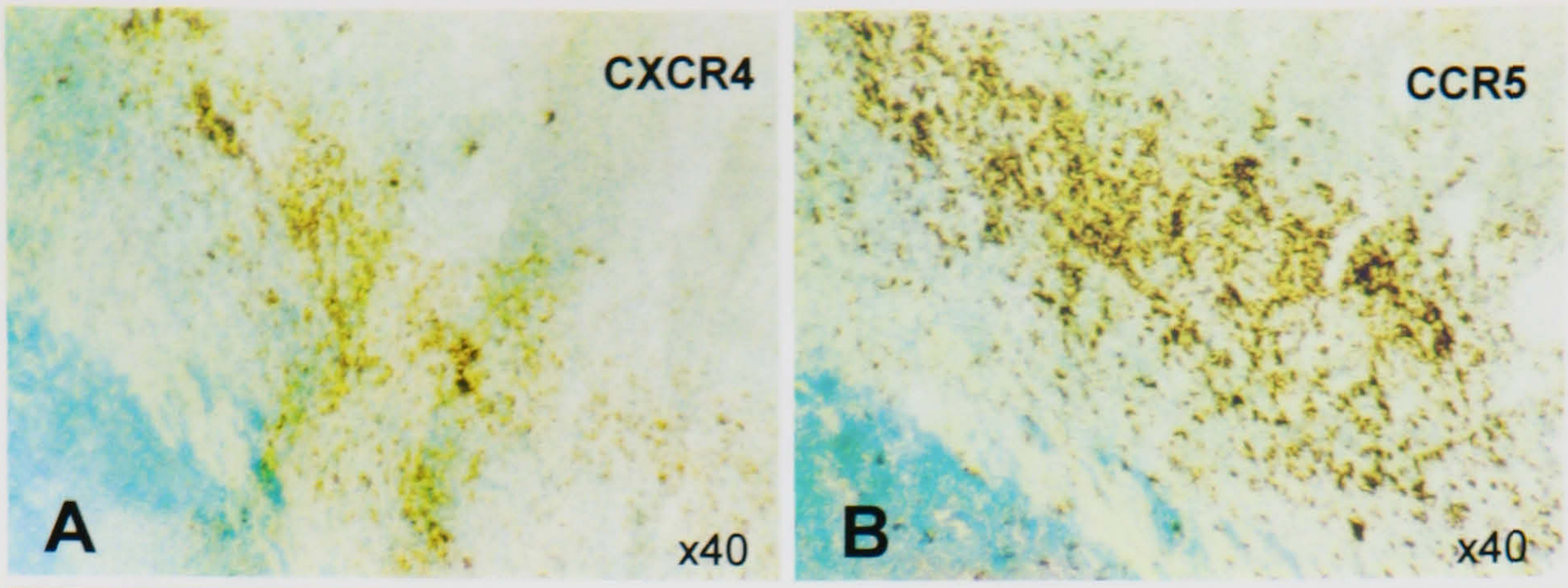


FIGURE 47

Expression of CXCR4 and CCR5 in human foetal nervous system

(**A**) CXCR4 was expressed on cells within the subventricular zone and extensions of the corpus callosum in the cerebrum. (**B**) CCR5 was heavily expressed in the same region, and more widely distributed in the germinal layer and intermediate zone. (**C,D**) CXCR4 expression was more localised within the human foetal spinal cord in the first timester, and likely to coincide with waves of neuronal migration in these areas.

Human foetal cerebrum. A: CXCR4 (brown) expression in the subventricular zone/ corpus callosum (the germinal layer is to the lower left of the figure), 22GW frontal cortex. B: CCR5 (brown) expression in the subventricular zone/corpus callosum (germinal layer to the lower left of figure), 22GW frontal cortex. **Human foetal spinal cord**. C: CXCR4 (brown/black) expression is localised in the spinal cord at 9GW, longitudinal section. D: higher magnification of (C) within the area of most intense CXCR4 expression (probably cervical/thoracic segment) at 9GW. Nuclei counterstained with methyl green. 30-40μm cryostat sections.

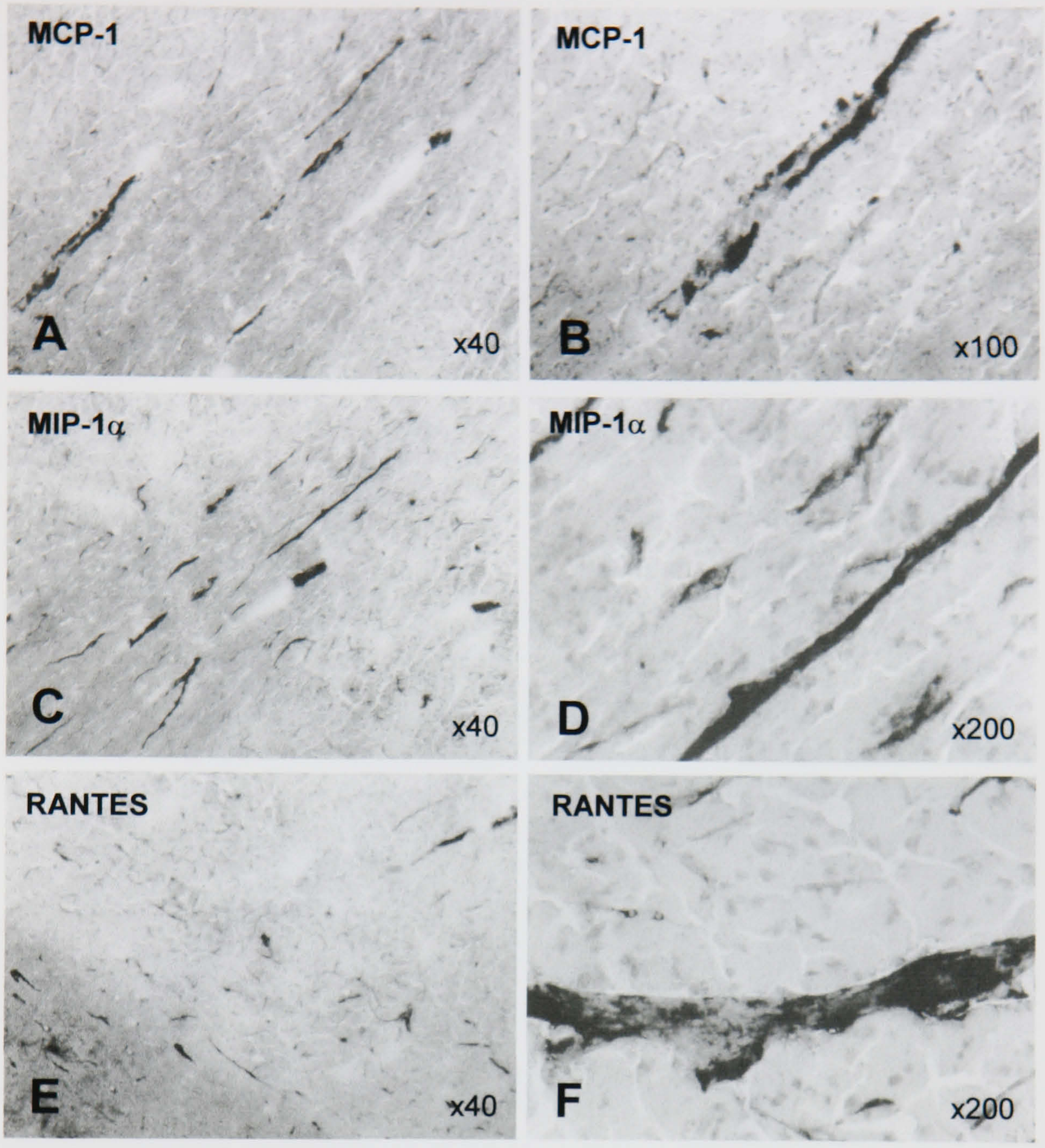


FIGURE 48

Expression of MCP-1, MIP-1α and RANTES in normal infant brain

In the normal infant brain, MCP-1, MIP-1α, and RANTES were all confined to cerebral vessels (**A-F**). Expression of MCP-1(**B**) and RANTES (**F**) appeared discontinuous and patchy by comparison with that seen for MIP-1α (**D**). Close examination of this type of immunoreactive pattern suggested that MCP-1 and RANTES were most likely expressed by perivascular-associated cells (i.e.perivascular macrophages), whereas that for MIP-1α was probably endothelial expression, although this needs to be confirmed.

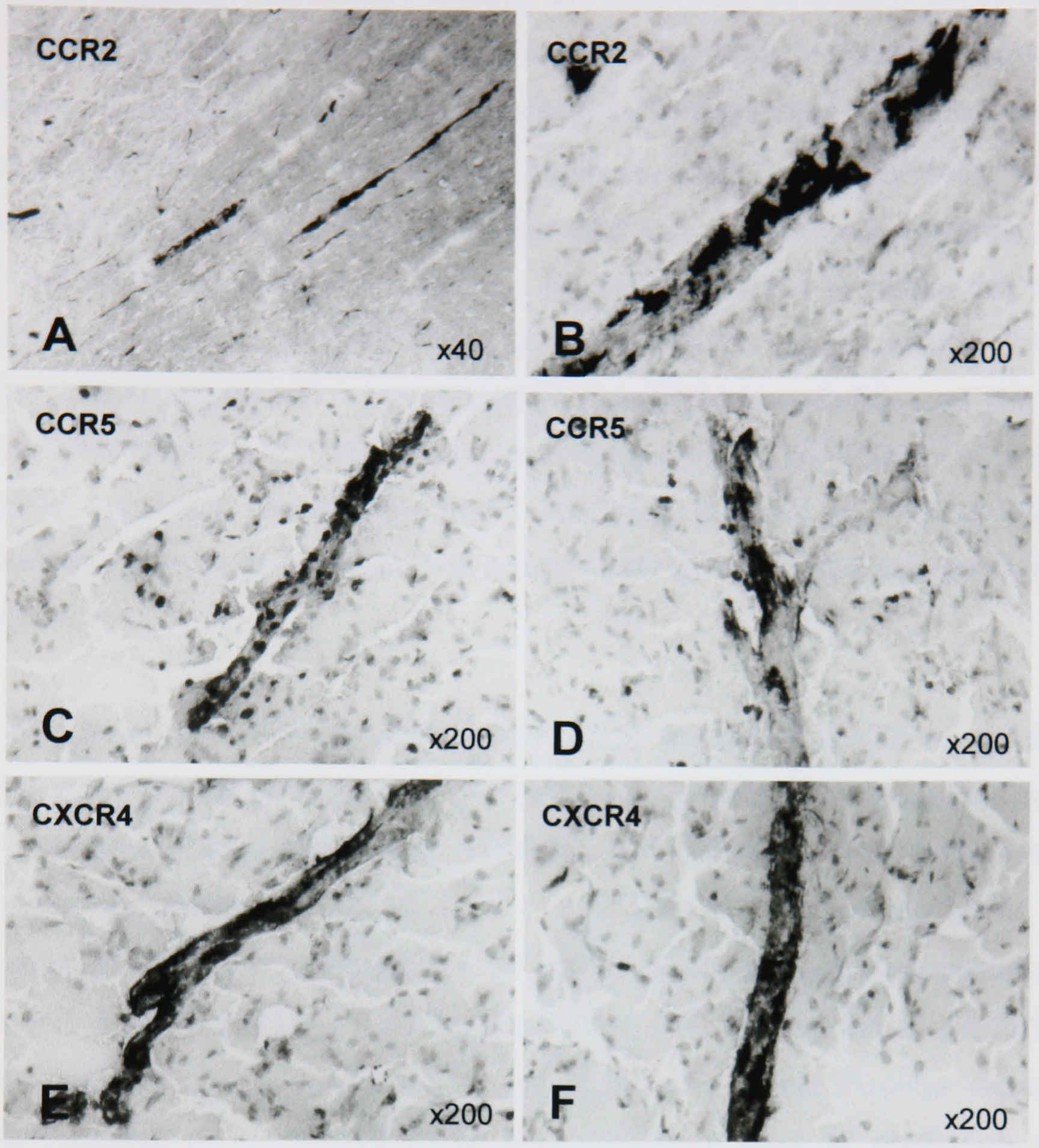


FIGURE 49

Expression of CCR2, CCR5 and CXCR4 in normal infant brain

CCR2 (**A,B**) and CXCR4 (**E,F**) were also confined to cerebral vessels in the normal infant brain. By contrast, other cells in the vicinity of blood vessels were immunoreactive with CCR5 (**C,D**), although this chemokine receptor was additionally expressed in patches on endothelium. Here, once more, the nature of this pattern of immunoreactivity suggested that perivascular cells were being identified through their known expression of these chemokine receptors. This hypothesis remains to be confirmed.

Although microglia appeared morphologically and numerically diminuitive in two out of three cases of Down's syndrome examined, when compared regionally to normal foetal brains, they nevertheless did express CCR2 receptors and MIP-1α similar to that which occured normally (personal observations). MIP-1α in these brains however, was additionally but weakly immunoreactive on vessels within the intermediate zone (personal observations). Although these observations are important, there was insufficient material to pursue this avenue of investigation further in cases of Downs syndrome. Our positive control material also included material from a neonate with acute-on chronic ventriculitis/encephalitis, and this case had remarkably elevated expression of chemokine receptors CCR2 and CXCR4 on a variety of cell types within the cortical grey matter (including neurons), as well as on cerebral endothelium, particularly upregulated on reactive cells that populated the ependymal lining (not shown).

Since the earlier studies had highlighted the potential importance of MIP-1α and MCP-1 in directing microglial colonisation, *in situ* hybridisation was performed on adjacent sections of frozen material, to demonstrate where, regionally these chemokines were being synthesised. Oligonucleotide probes directed at MCP-1 and MIP-1α, revealed expression of their respective mRNA within the ventricular zone (MCP-1, MIP-1α), ganglionic eminence (MCP-1) and more diffusely within the cortical plate (MCP-1, MIP-1α) between 17 and 22GW in the normal human foetal brain (**Figure 50** and **Figure 51**). These findings supported the immunohistochemical studies indicating that the major sites of production of these chemokines were located within the ventricular zone/germinal layers (including the ganglionic eminence where neuroglial progenitors prevailed), and within the cortical plate (where neuronal progenitors where prevalent). There were insufficient materials and resources to pursue this most interesting finding further during the course of this investigation. However, since the results presented here have emphasised that the corresponding patterns of protein expression (determined immunohistochemically) for Fractalkine and RANTES, and the chemokine receptors CCR2, CCR5 and CXCR4, appear equally significant from the perspective of microglial colonisation, future work should determine where regionally, the mRNA for these and other chemokines and chemokine receptors are expressed within the human brain during the second trimester.

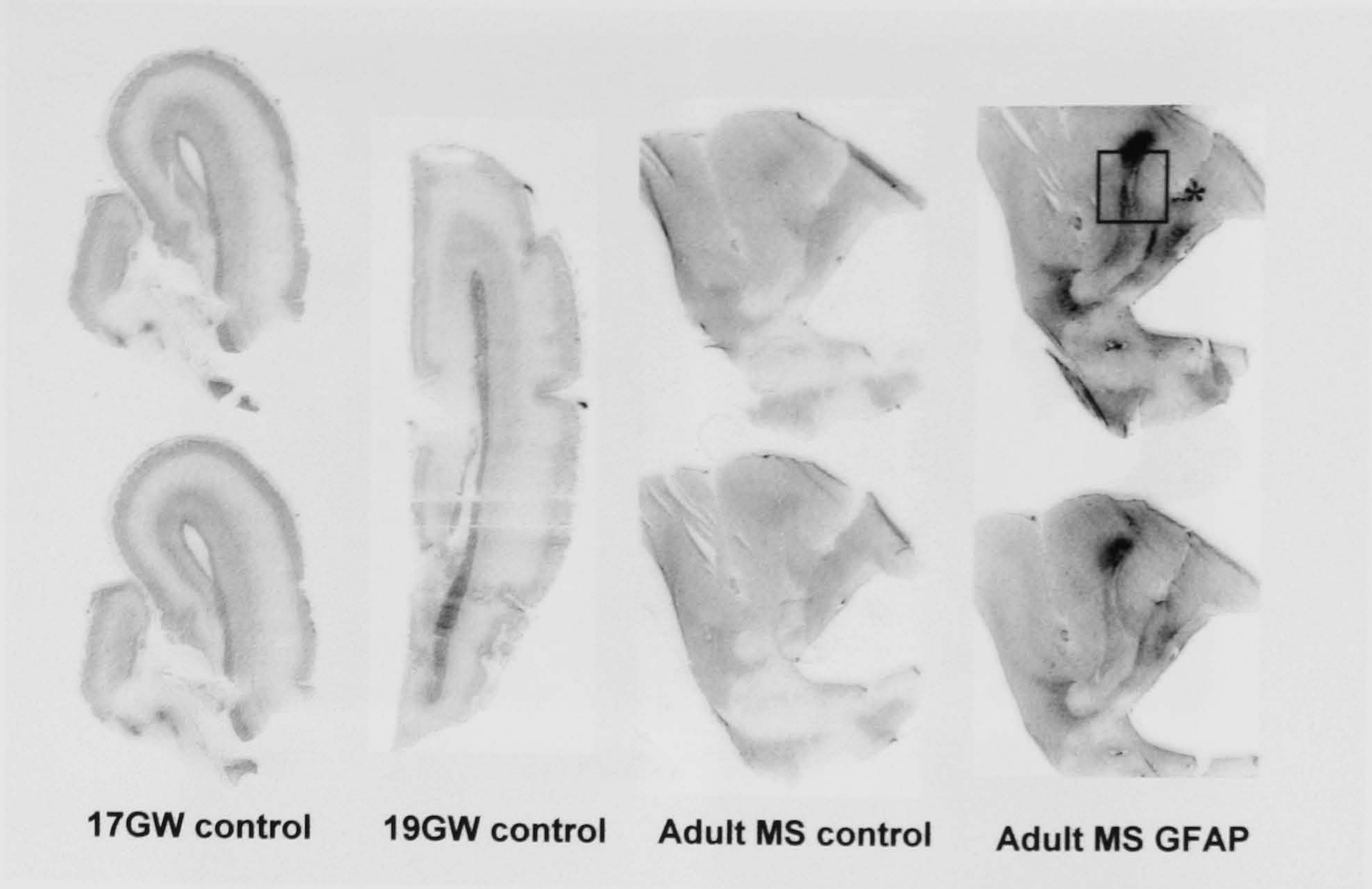


FIGURE 50

In situ hybridisation for MIP-1α and MCP-1 mRNA on frozen sections of the human foetal telencephalon 17-22GW

(**A**) 17GW and adult multiple sclerosis (MS) control sections were incubated with hybridisation buffer lacking probe (top panel of duplicate sections) or incubated with unlabelled probe (lower panel of duplicate sections). The 19GW control section was incubated with hybridisation buffer lacking probe. The positive control MS sections were incubated with an oligonucleotide probe to GFAP. (**B**) Test sections of the human foetal brain incubated with MIP-1α and MCP-1 oligonucleotide probes. Note the intense labelling of the ventricular zone with MIP-1α at 17GW, MCP-1 at 19GW and diffusely within the cortical plate at 17GW and 22GW with MCP-1. At 22GW, the ganglionic eminence (GE) is intensely labelled with MIP-1α and MCP-1 mRNA. (*) see Figure 51A/B, (**) see Figure 51D, (***) see Figure 51F. No counterstain.

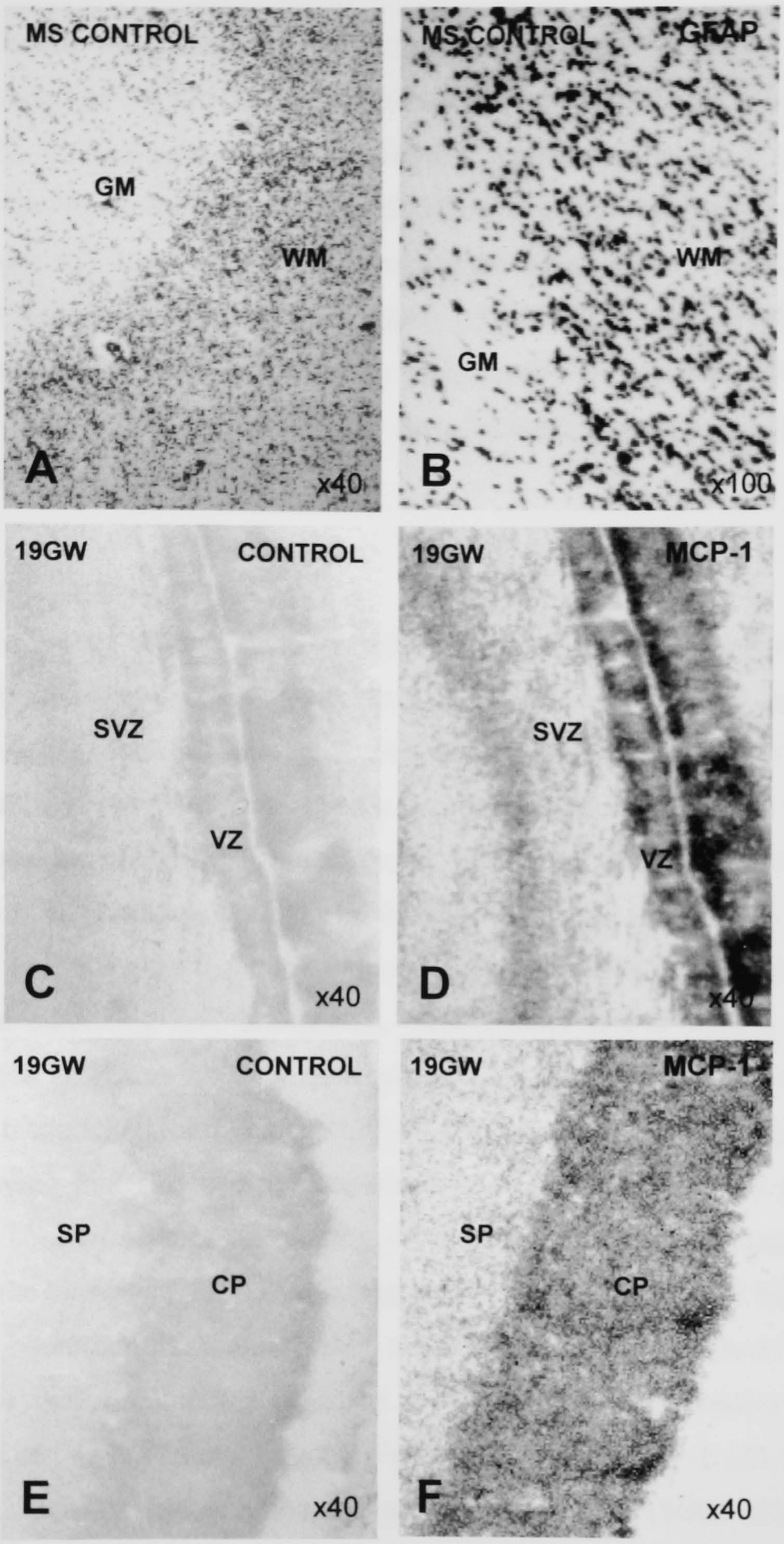


FIGURE 51

***In situ* hybridisation for MCP-1 and GFAP mRNA**

Images correspond to sections shown in Figure 50. (**A,B**) Sections of the CNS from a case with multiple sclerosis labelled with an oligonucleotide probe to GFAP as control. Note the intense labelling, particularly within the white matter (WM).(**C,E**) Negative control sections incubated with hybridisation buffer lacking probe. (**D,F**) Non-radioactive *in situ* hybridisation with an oligonucleotide probe to MCP-1showing diffuse labelling within the cortical plate (CP) and more intense labelling within the ventricular zone (VZ) between 19 and 22GW. GM: grey matter; WM: white matter; CP: cortical plate; SP: subplate; VZ: ventricular zone; SVZ: subventricular zone. No nuclear counterstain.

Apoptosis

Human foetal brains were screened to determine the regional extent of apoptosis in the telencephalon using the TUNEL method (Gavrieli et al. 1992). The most significant finding was that TUNEL-labelled cells were predominantly interspersed within the ventricular zone and subventricular zones between 19 and 22GW where they inevitably co-localised with microglia (**Figure 52**). However, other regions of the telencephalon where microglia could also be located, lacked any appreciable labelling with TUNEL. These findings suggest that apoptotic cell death in the SVZ and VZ may be important for recruiting foetal microglia to these particular sites, but does not explain the more widespread and progressive dissemination of these cells throughout the brain.

Stamper-Woodruff assay

In attempting to pursue these lines of thought further, and to determine whether potential microglial progenitors (human monocytes) can bind to blood vessels within specific regions of the brain (i.e. points of entry for microglial progenitors), sections of human foetal brains were overlaid with cells derived from the human monocytic cell line THP-1, according to the procedure outlined under the methods section, modified from Stamper and Woodruff (1976). THP-1 cells could be identified in culture with markers directed against CD11b, CD45, CD68 or with RCA-1 (not shown). The results presented in **Figure 53**, **Figure 54**, and **Figure 55** were somewhat surprising and unexpected. THP-1 cells bound with a high degree of specificity to the subplate, lower cortical plate and intermediate zone, and to a region overlying the marginal layer (**Figure 53C-F**). Clearly this binding was dependent on interaction between integrins and extracellular matrix components since preincubation of sections with a cocktail of peptides blockng laminin, fibronectin and collagen binding sites, interfered with the binding of THP-1 cells (**Figure 53A,B**). The pattern of THP-1 cell binding to human foetal brains correlated closely with the expression of MCP-1 (and RANTES) within this site, and to the accumulation of foetal microglia and differentiating GFAP positive astrocytes presented earlier in this Chapter. Further analysis of THP-1 cell binding revealed them to interact specifically with the sub-pial granular layer (SGL) (**Figure 54A,B**), a transitory structure in man that is present in the latter half of the second trimester (H. Uylings, personal communication). The SGL consists of neurons (derived from the retrobulbar ventricle) and glia (derived from the neocortical ventricular zone), and is thought to be important for the proper migration of cortical neurons and their formation into layers, a phenomenon that cells of the SGL may co-ordinate through interaction with Cajal-Retzius cells (H. Uylings, personal communication).

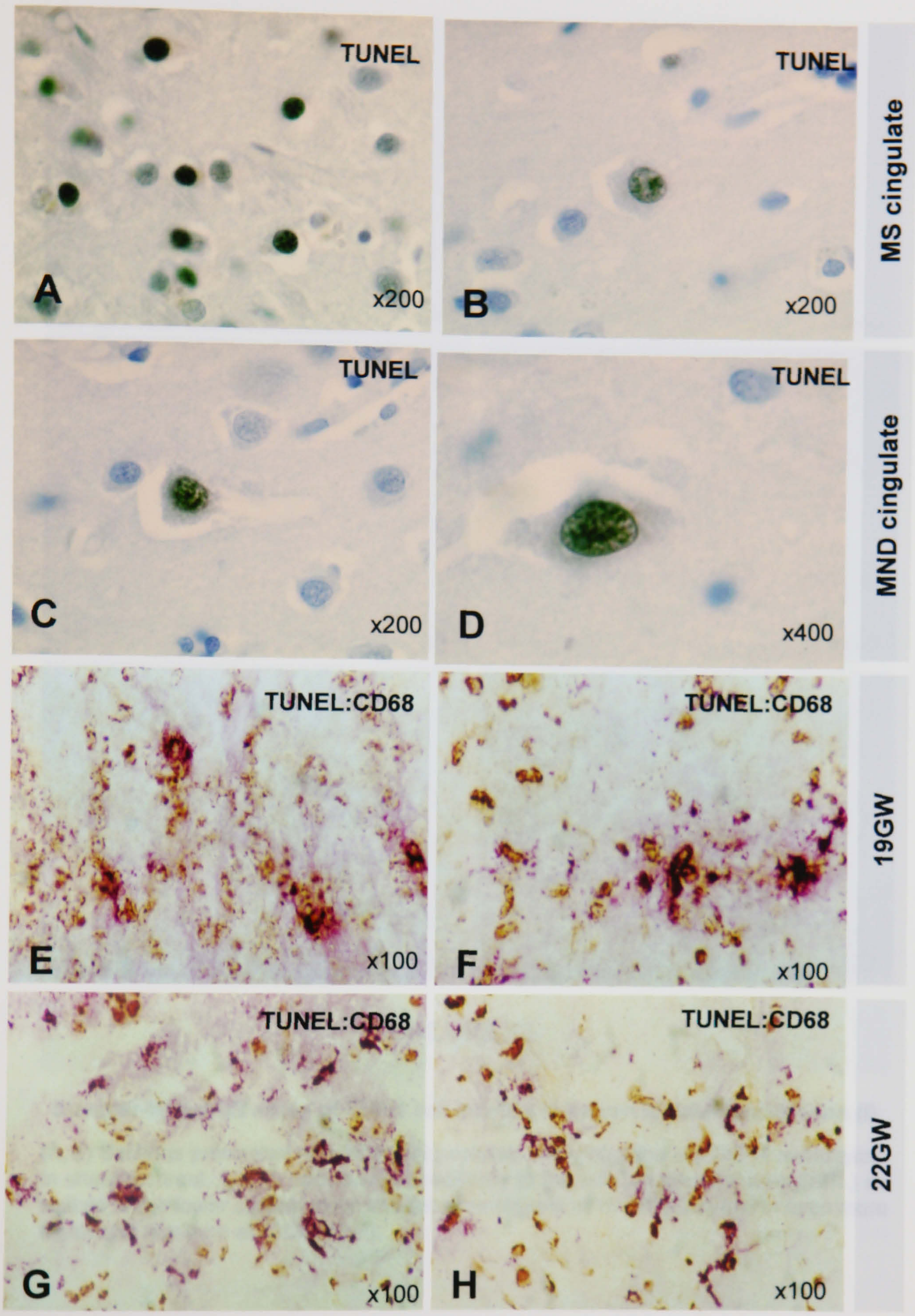


FIGURE 52

TUNEL reactivity in positive control tissues and within the human foetal subventricular zone 19-22GW

(**A,B**) TUNEL reactivity in the cingulate cortex from an adult case with multiple sclerosis. (**C,D**) TUNEL reactivity in the cingulate cortex from a case with motor neuron disease. TUNEL labelling within the human foetal subventricular zone at 19GW (**E,F**) and 22GW (**G,H**), co-localised with microglia. A-D: TUNEL reaction alone; E-H: TUNEL reaction (brown), CD45/68 (violet).

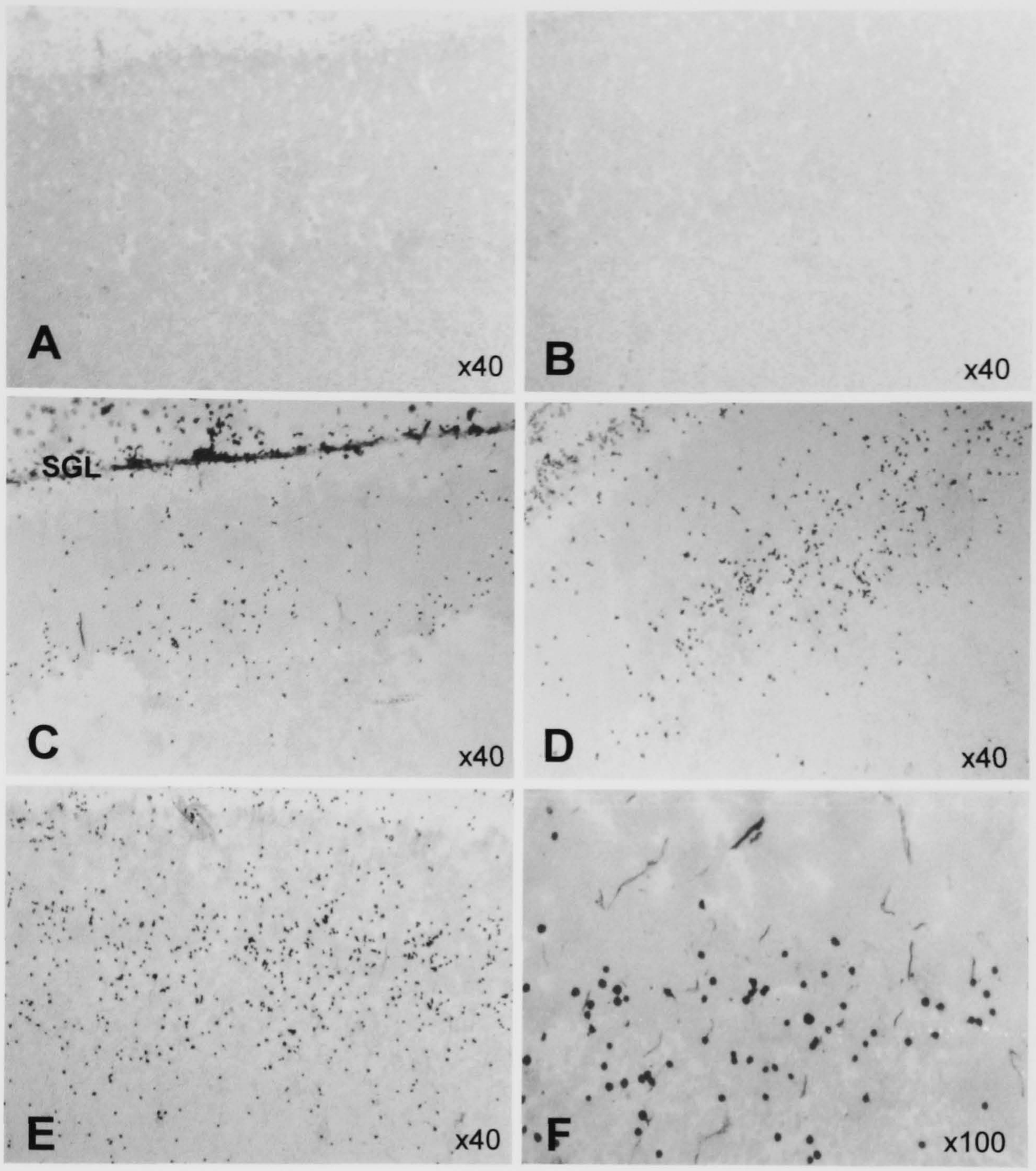


FIGURE 53

Stamper-Woodruff assay of THP-1 cell binding to human foetal telencephalon (I)

(**A,B**) Sections pretreated with inhibitory peptides, show no adherent THP-1 monocytes. In sharp contrast, THP-1 cells bound specifically to the sub-pial granular layer (SGL) as well as to the lower cortical plate and subplate regions at 18GW (**C,D**) and in even more significant numbers at 22GW (**E-F**).

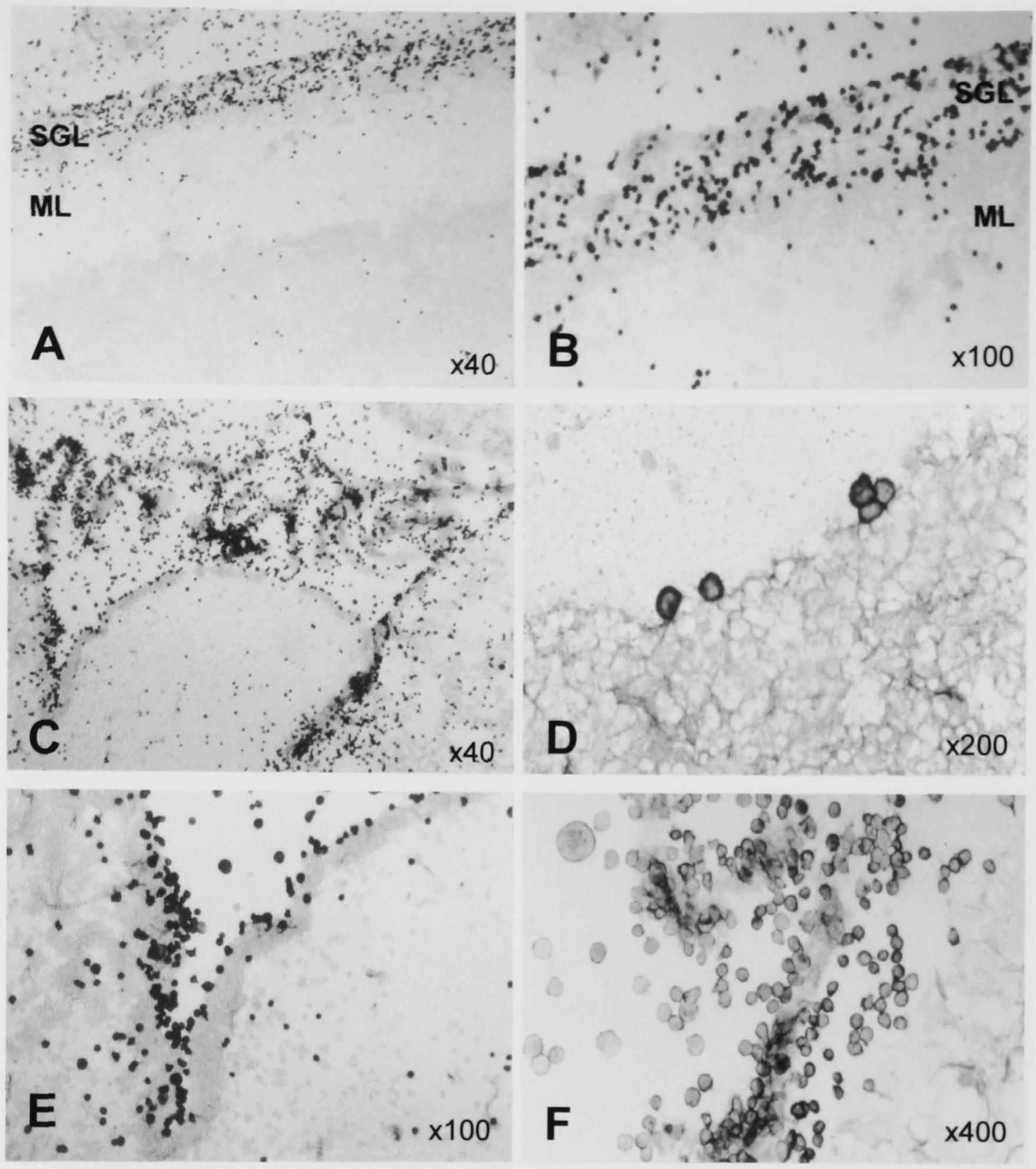


FIGURE 54

Stamper-Woodruff assay of THP-1 cell binding to human foetal telencephalon (II)

(**A,B**) A large number of of THP-1 cells adhered to the sub-pial granular layer (SGL) overlying the marginal layer of the telencephalon at 19GW. (**C,D**) Adherent cells were frequently detected within the choroid plexus (**C,F**) and attached to the ependymal layer lining the lateral ventricles (**C-E**). Few cells were found scattered throughout the parenchyma of the telencephalon (**A,C,E**). A,B: 19GW, C,E,F: 17GW, D: 22GW

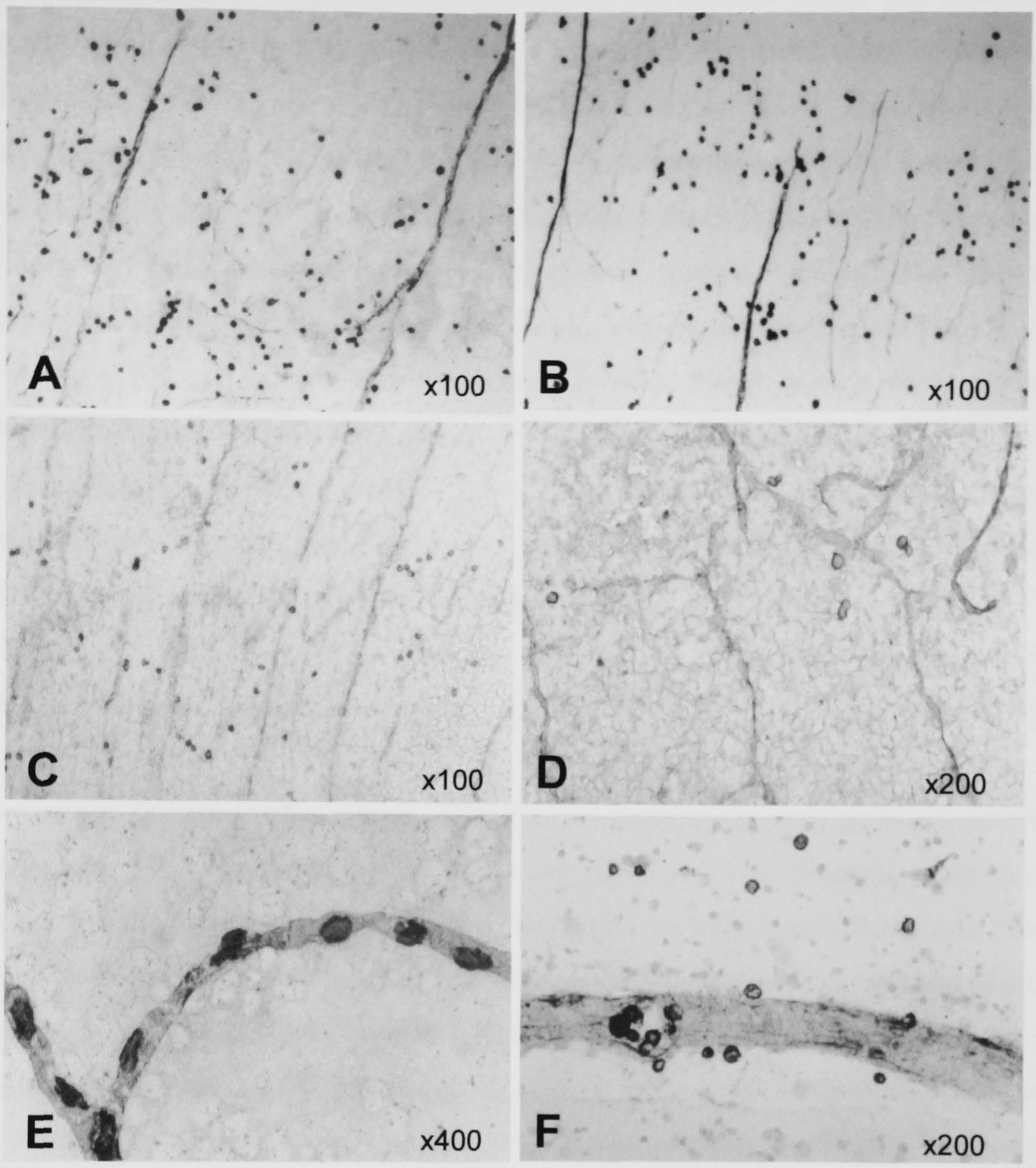


FIGURE 55

Stamper-Woodruff assay of THP-1 cell binding to human foetal telencephalon (III)

(**A-D**) THP-1 cells were found to adhere strongly to the parenchyma of the intermediate zone within the telencephalon at 23GW (A,B) less so at 19GW (C) or 20GW (D). Bound cells were frequently located to perivascular sites, but rarely adherent to cerebral endothelium of radiating cortical vessels. (**E**) Occasionally, adherent cells were identified closely associated with small capillaries in the intermediate zone at 19GW. These were spatially distributed along the length of the vessel, in a similar pattern to that seen with perivascular cell investment of cerebral blood vessels. (**F**) In positive control CNS tissue from a case with multiple sclerosis, THP-1 cells were clearly found to adhere to perivascular sites and both ablumenal and lumenal aspects of cerebral vessels, as shown. A,B: 23GW; C: 19GW, D: 20GW; E: 19GW; F: adult MS case

Adherent cells were also frequently detected within the choroid plexus and attached to the ependymal layer lining the lateral ventricles (**Figure 54**). Few cells could be seen scattered randomly throughout the rest of the CNS parenchyma prior to 22GW. By contrast, at 23GW. THP-1 cells were found to adhere strongly to the parenchyma of the intermediate zone (**Figure 55A-D**). Although bound cells were frequently located to perivascular sites, they were rarely adherent to cerebral endothelium of radiating cortical vessels. Very infrequently, adherent cells could be identified distributed along small capillaries (**Figure 55E**) in a similar array as seen for perivascular cells investing cerebral blood vessels (data not shown). However, these were few and far between and did not, on the whole, coincide closely with areas populated by microglia. THP-1 cells were clearly found to adhere not only at perivascular sites, but also to the lumenal and ablumenal aspects of cerebral vessels in CNS tissue from a case with multiple sclerosis (**Figure 55F**).

VI. Microglia in the human foetal spinal cord

Sections of the thoraco-lumbar region of the human foetal spinal cord derived between 9 and 16 weeks menstrual age (here also referred to as GW) from nine foetuses, were assessed to determine the temporo-spatial patterns of colonisation by microglial progenitors (**Table 4**). Spinal cords from the lumbar region of five normal adults (negative controls) and two cases of motor neuron disease (positive controls) were also screened (**Table 5**). Between 9 and 16 weeks, foetal microglia of the spinal cord presented as a heterogeneous population of cells, with subsets identifiable with monocyte/macrophage markers (**Table 9**). Differential expression of the other markers employed, are also indicated in this table for comparison.

Table 9. Differential expression of markers in human foetal spinal cord 7-15 weeks*

Marker	7-8	9	10-11	12-13	14-15	MND	Normal
CD11b	nd	+	-	-	+	++	-
CD45	+	±	±	-	+	++	-
CD64	nd	-	-	-	+	+	-
CD68	+	+	++	++	++	+++	-
CD45:68	++	++	+++	+++	+++	+++	-
HAM-56	nd	-	±	-	+	+	-
RCA-1	-	-	nd	-	+	+	-
GFAP	++	++	nd	++	++	++	-
Vimentin	++	++	nd	++	nd	-	-
ICAM-2	-	±	++	++	++	-	-
PECAM-1	++	++	++	++	++	±	±

Key: (-) no staining, (+) weak positive staining, (++) positive, (+++) intense staining; (nd) not determined
A minimum of ten consecutive sections were analysed from each sample
(*) Foetal age given in weeks from conception/fertilisation

At the earliest time point, microglial progenitors were preferentially located around the ependymal layer surrounding the neural cavity (**Figure 56A,B**). These associated with vimentin-immunoreactive radial glial processes within the same region and vimentin-positive radial glial fibers could be seen to extend outwards from the ventricular zone through the marginal layer of the cord to the periphery (**Figure 56C,D**). These initial foetal microglia were predominantly amoeboid and few in numbers (totalling between 10 and 20 CD45:CD68 positive cells per 40-60μm section). Immunoreactivity with vimentin further demonstrated a dense meshwork of radial glial fibers oriented within the marginal layer, and a numerous network of vimentin-positive mesenchymal cells (the majority possessing bipolar and elongated morphologies, distinct from macrophages, also present within this region- refer to **Figure 58** and **Figure 59**) in the connective tissue surrounding the spinal cord (**Figure 57**). Macrophages demonstrated using combined immunohistochemistry with CD45 and CD68, could be detected within the vertebral column and connective tissues surrounding the spinal cord from 11 weeks (**Figure 58** and **Figure 59**). These samples were also screened for expression of adhesion molecules, chemokines and chemokine receptors as previously outlined. Of these, ICAM-1, ICAM-2 and VCAM-1 were not expressed at 9 weeks. At 12-13 weeks, ICAM-2 was expressed on vessels at the periphery of the spinal cord, and co-localised with macrophages in the connective tissue (**Figure 59**). Neither ICAM-1 nor VCAM-1 immunoreactivity were detectable. Within the spinal cord, CD45:CD68 positive cells were migrating inwards from the ventral (floor plate) and dorsal (roof plate) aspects of the cord into the ventral and dorsal funiculi respectively, between 14 and 16 weeks (**Figure 60**). Whereas PECAM-1 detected the majority of blood vessels within the spinal cord (**Figure 60**) much like that seen in the cerebrum, ICAM-2 showed the microvasculature of the cord clearly at 14 weeks and was notably expressed on vessels situated at the periphery, which supplied both dorsal and ventral aspects of the thoraco-lumbar region (**Figure 60**). By 16 weeks, the colonisation of microglial progenitors was in full progress within the spinal cord, and the influx of cells dorsally and ventrally (with corresponding densities of 40-50 cells/mm^2), was associated closely with delicate vessels that specifically expressed ICAM-2 within the same regions (**Figure 61**). Other regions of the spinal cord (dorso-lateral, medial and ventro-lateral) totalled between 5-30 CD68+ cells/mm^2 at 16 weeks. Interestingly, the pattern of reactivity with GFAP at this time, demonstrated astrocytes and their processes preferentially localised to the marginal layer and their processes no longer spanning the breadth of the spinal cord (data not shown). This arrangement of fibers, appeared to preferentially favour the inward migration of microglial progenitors. With the exception of CXCR4 (shown in **Figure 47C,D**) somewhat surprisingly, and in sharp contrast to the human foetal brain, none of the chemokines or chemokine receptors analysed were found to be expressed within the human foetal spinal cord from 9-16GW.

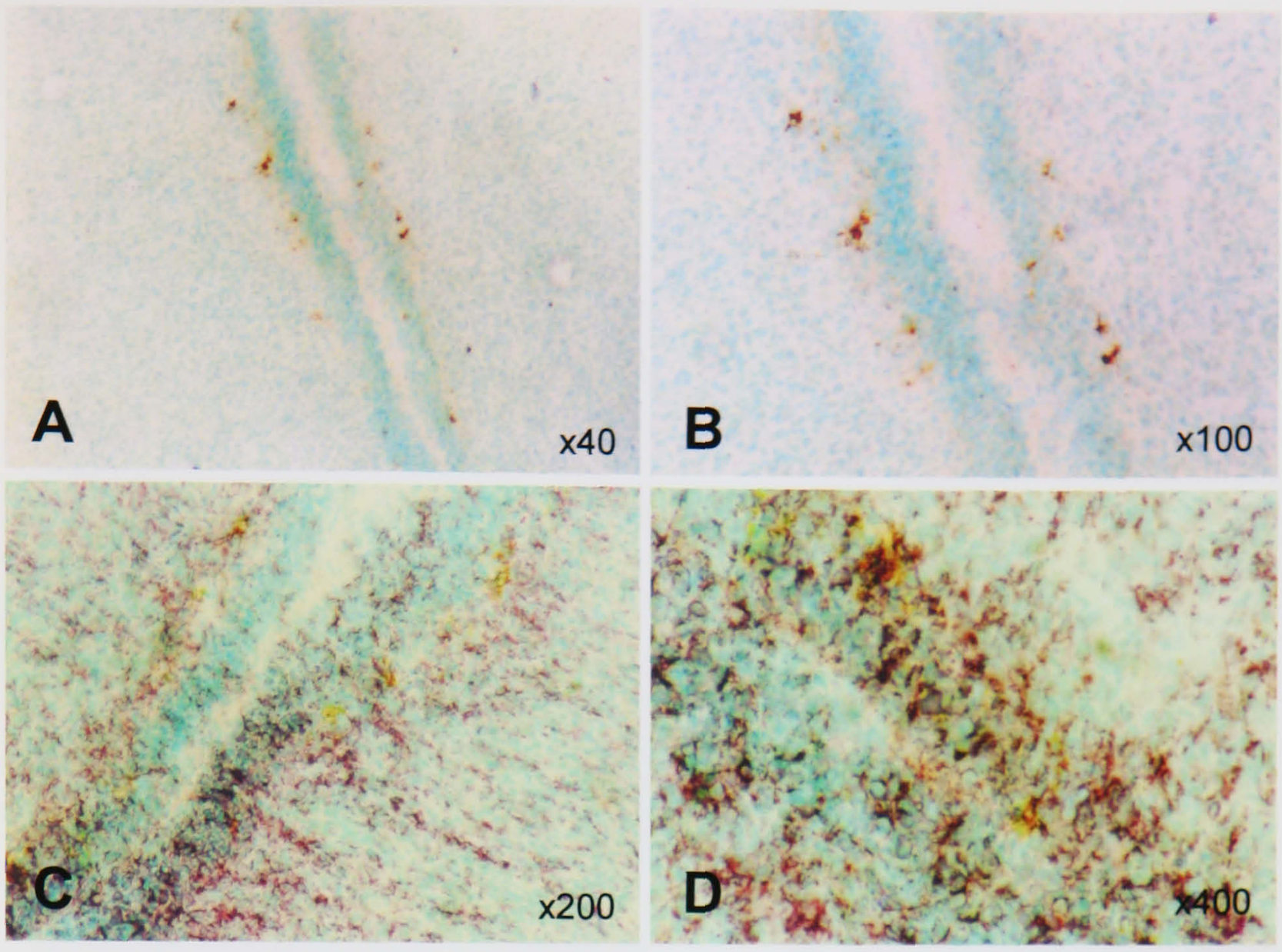


FIGURE 56

Foetal microglia and radial glia in human spinal cord, 9-10GW

(**A,B**) Microglia are preferentially located around the ventricular layer surrounding the neural cavity at this stage in development (CD45:CD68); (**C,D**) Figures to demonstrate microglia (CD45:CD68, brown) and vimentin-positive radial glial processes (violet) arising from the ependymal layer (these radial glial fibers are arranged in parallel , and span the region between the ependymal layer and the marginal layer of the cord (refer to Figure 99 for anatomy of the foetal spinal cord).

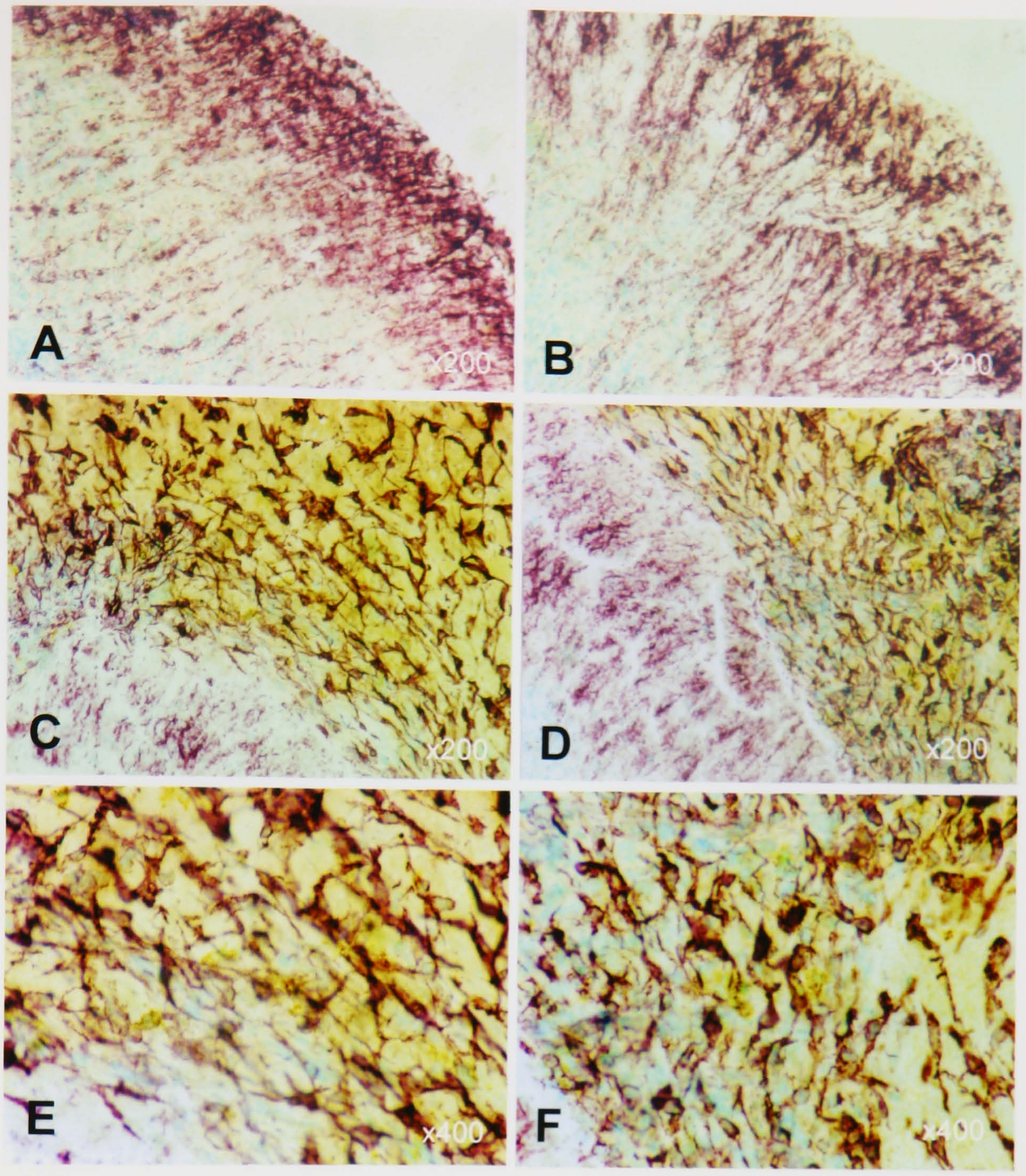
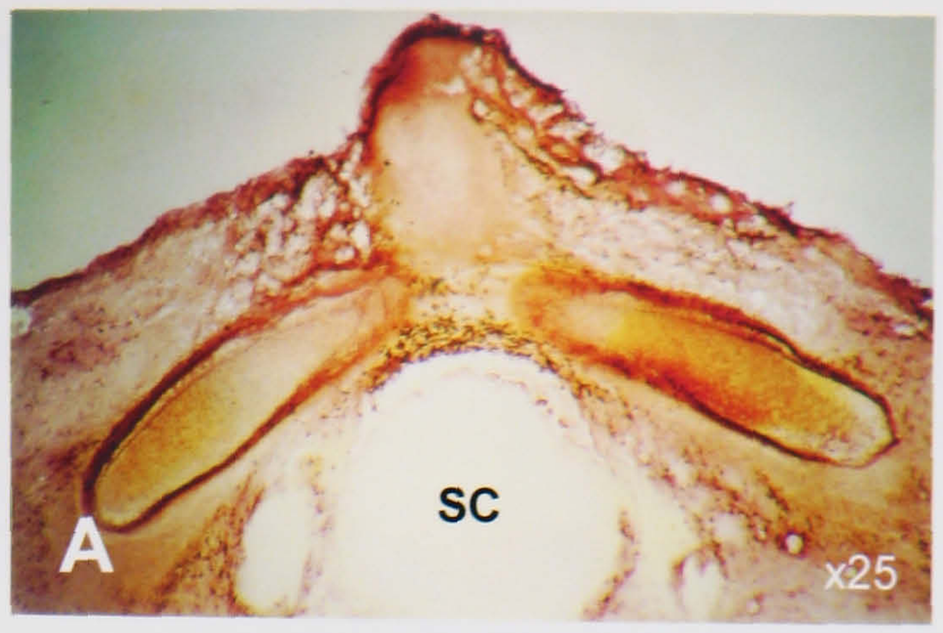


FIGURE 57

Vimentin immunoreactivity associated with the human foetal spinal cord

(**A,B**) Immunoreactivity with vimentin (violet) detects a dense meshwork of radial glial fibers in the marginal layer between 9-10GW. (**C-F**) Within the connective tissue surrounding the spinal cord, there are numerous vimentin-positive mesenchymal cells which form a network. The majority of these cells are elongated or bipolar in morphology, and quite distinct from mononuclear phagocytes.



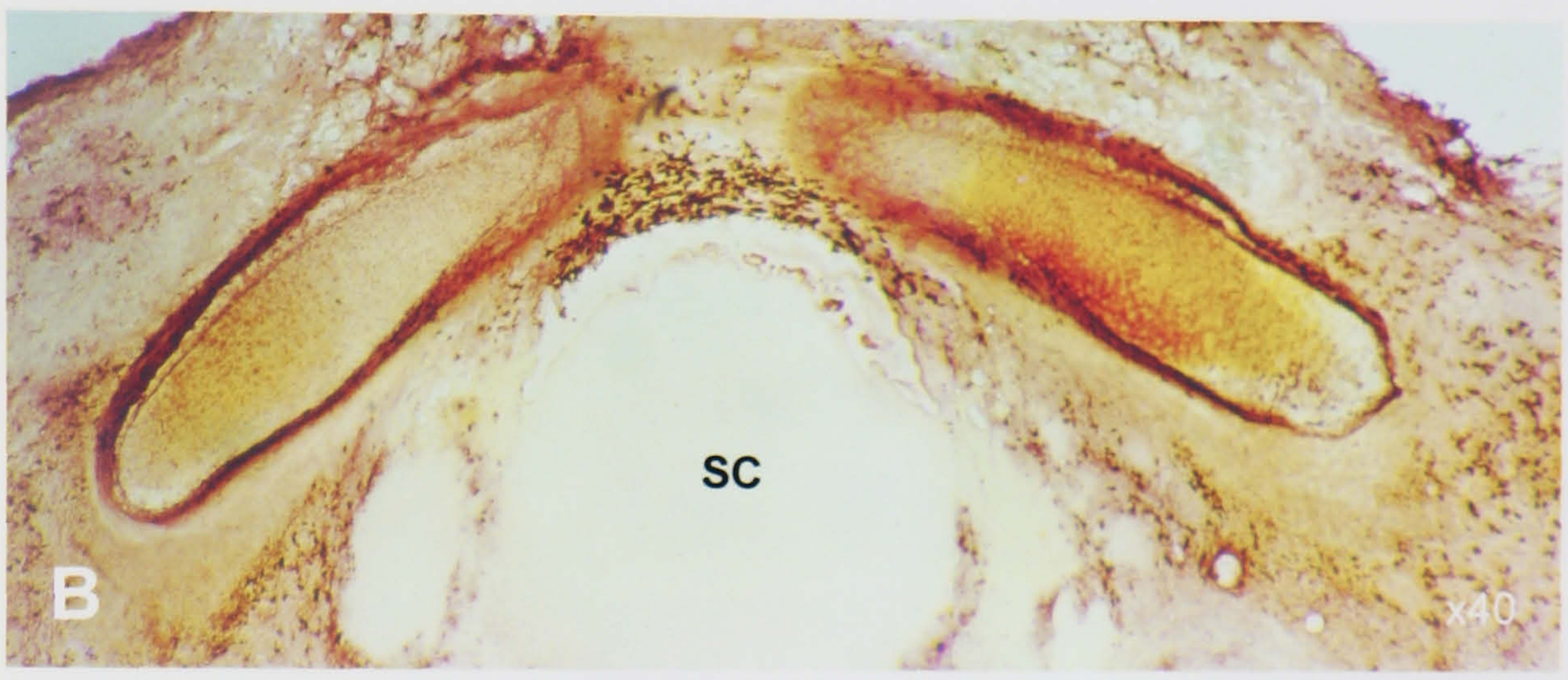


FIGURE 58

Macrophages surrounding the human foetal spinal cord, 12-13GW

Photomicrographs demonstrating combined CD45:CD68 immunoreactive macrophages (brown) within the vertebral column and connective tissues surrounding the spinal cord (SC)

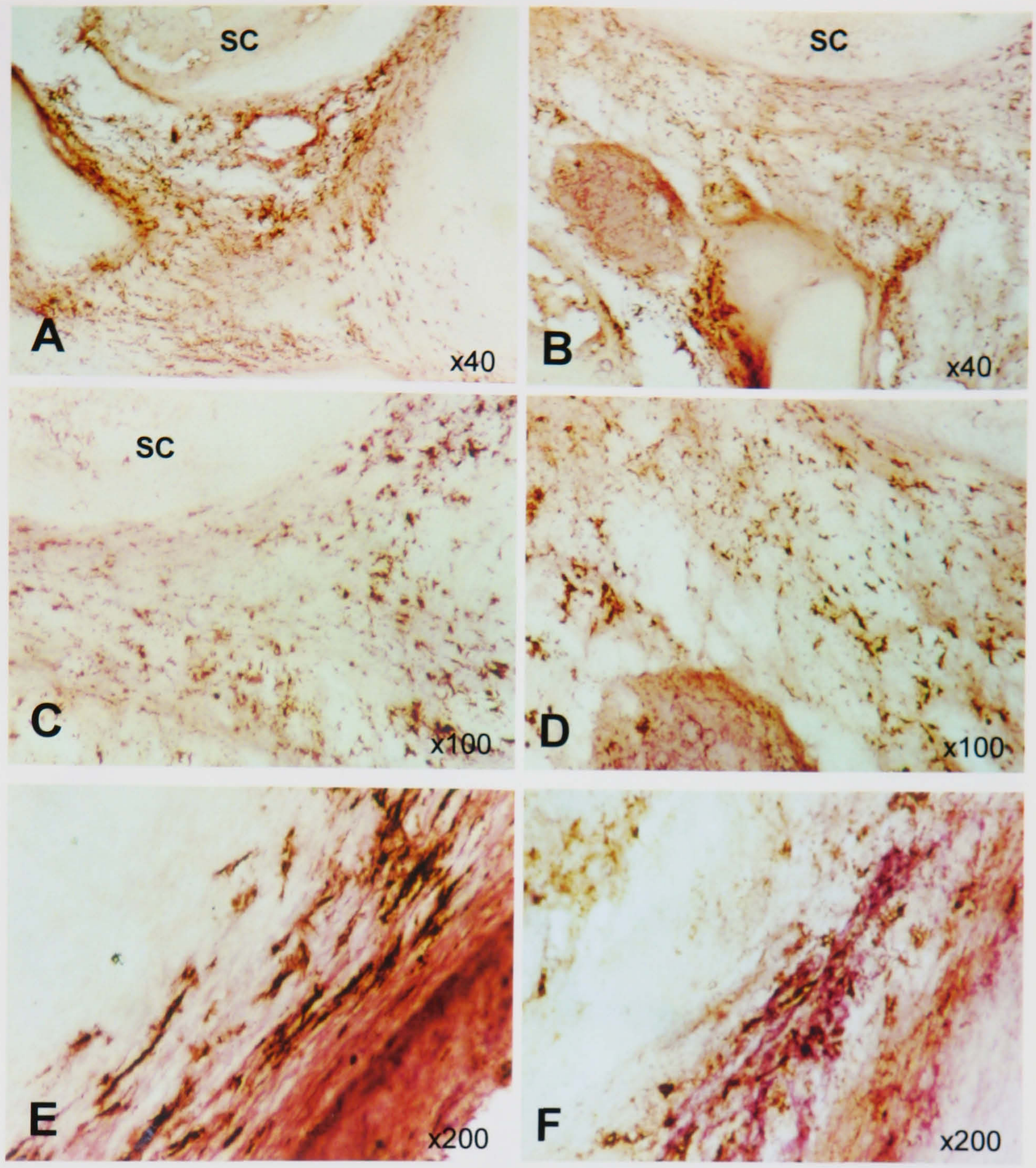


FIGURE 59

Macrophages associated with the spinal cord, 12-13GW

(**A-D**) Distribution of combined CD45:CD68 positive macrophages in the connective tissues surrounding the spinal cord, 12-13GW. (**E,F**) At the periphery of the spinal cord, macrophages (brown) are associated with blood vessels expressing ICAM-2 (violet).

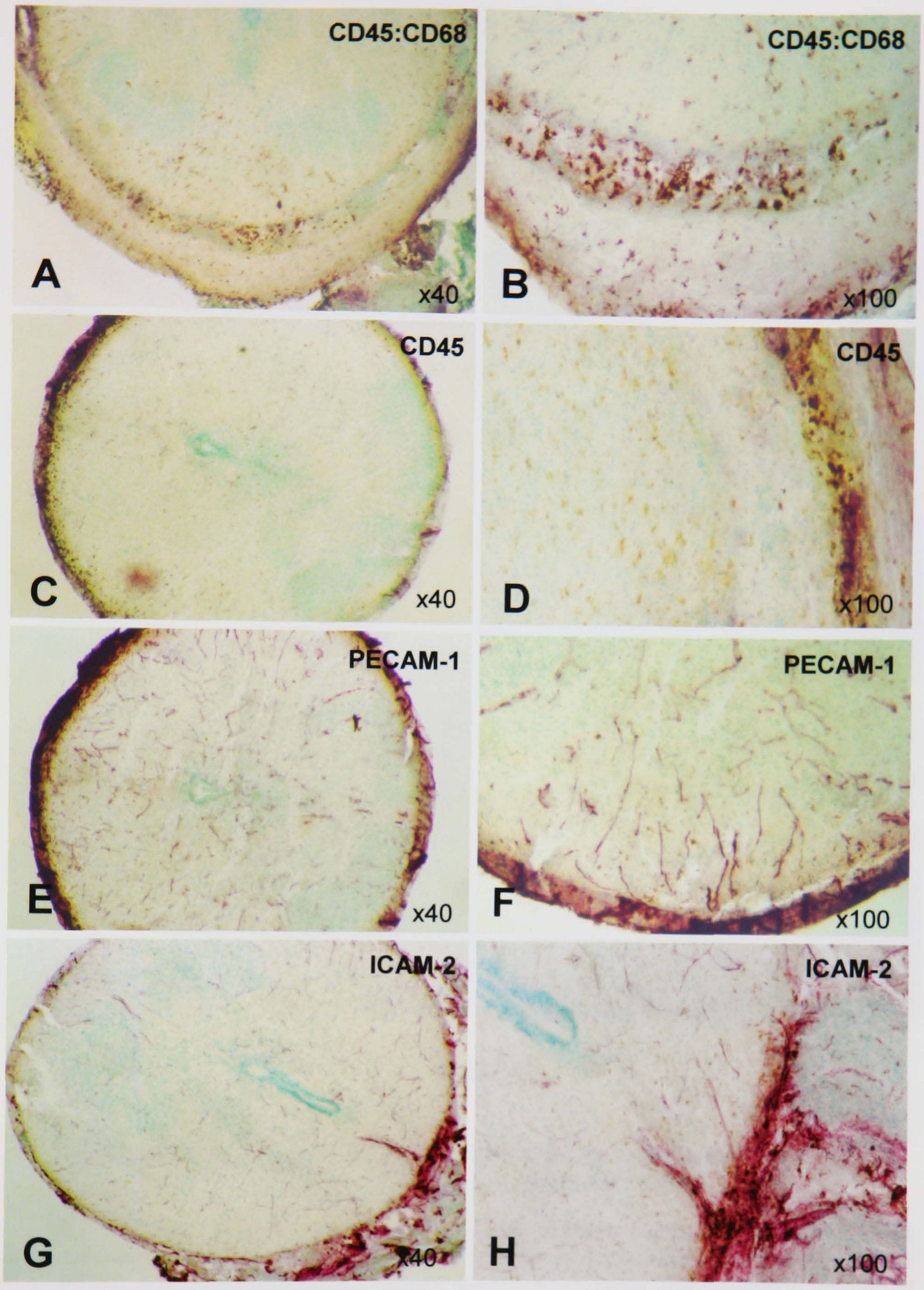


FIGURE 60

Macrophage distribution, PECAM-1 (CD31) and ICAM-2 expression in the human foetal spinal cord, 14-16GW

(**A,B**) Low power (A) and higher power (B) figures showing the distribution of combined CD45:CD68 immunoreactive macrophages. Note the high density of immunolabelled cells surrounding the spinal cord and migrating inwards within the ventral aspect of the cord. (**C-D**) Immunoreactivity with CD45 shows a similar distribution of cells positioned around the cord (**C**) and within the region ventral to the neural cavity. (**E,F**) Immunoreactivity with PECAM-1 (CD31) demonstrates the majority of blood vessels within the spinal cord. (**G,H**) Immunoreactivity with ICAM-2 shows the microvasculature of the cord clearly (14GW), and ICAM-2 is particularly expressed on blood vessels situated at the periphery of the spinal cord, which supply both dorsal (**H**) and ventral aspects of the thoraco-lumbar region.

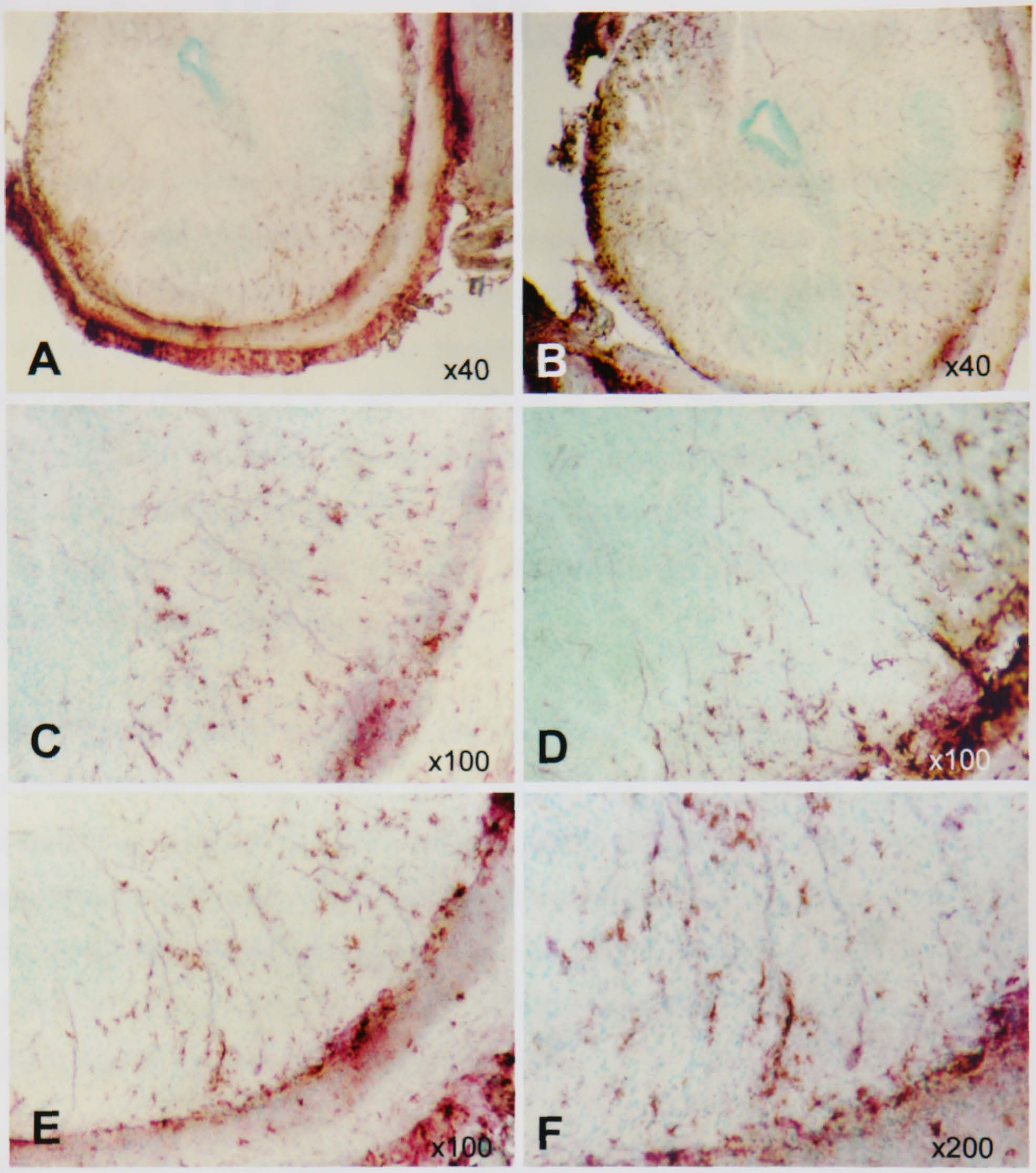


FIGURE 61

Microglial colonisation is associated with vessels expressing ICAM-2 in the ventral aspect of human foetal spinal cord

(**A,B**) low power figures to show the preferential distribution of combined CD45:CD68 immunoreactive foetal microglia within the ventral part of the thoraco-lumbar spinal cord at 16GW. This also occurred to a lesser extent within dorsal and peripheral regions of the cord (not shown). (**C-F**) Higher power dual-label photomicrographs demonstrate microglia (brown), associated closely with delicate vessels specifically expressing ICAM-2 within the region ventral to the neural cavity at 16GW.

Chapter III MICROGLIA IN THE FOETAL MOUSE BRAIN

From the studies presented thus far, we know that microglial colonisation of the human CNS takes place essentially during the second trimester. and commences from the beginning (and shortly before) this period. By comparison, studies in rodents have shown that microglia colonise the nervous system in the period just before birth, but mainly throughout the postnatal period (Dalmau et al. 1997; Miyake et al. 1984; Perry et al. 1985). The primary aim of this chapter is to define the corresponding timing, phases and extent of microglial colonisation that takes place in the mouse during the foetal period (embryonic day 15 to birth). This is necessary in order to establish similarities (or differences) in the modality of colonisation with that which occurs in man.

The phenotype of murine microglial progenitors and foetal microglia will be determined by lectin histochemistry using tomato lectin, RCA-1 and GSB4, and by immunoistochemistry with markers against CD11b, CD45, MOMA and F4/80 antigen. ICAM-2, MIP-1α and MCP-1 emerged among the potentially important factors regulating microglial colonisation in man. In order to compare these findings with the murine CNS, sections of foetal mouse brains were also screened for expression of vascular adhesion molecules PECAM-1, ICAM-1, ICAM-2, VCAM-1, and the chemokines MIP-1α and MCP-1.

The following points were addressed in relation to the developing murine CNS:

- What are the phases of colonisation and regional patterns of distribution of microglial progenitors within the foetal murine CNS between E15 and birth?
- Is ICAM-2 expressed in the murine foetal CNS as a potential signal for the recruitment of microglial progenitors? Are PECAM-1, ICAM-1 and VCAM-1 expressed on cerebral vessels during development in the mouse?
- What are the patterns of expression of MIP-1α and MCP-1 in the murine foetal CNS? How do these correspond to that seen during the second trimester of development in man?

MATERIALS & METHODS

Tissue samples and histological processing

Time-mated female Balb/c mice were purchased from Harlan Olac, UK (delivered on the 13th day of gestation) and sacrificed humanely by placement in a lethal chamber infused with carbon dioxide and chloroform, followed by rapid dislocation of cervical vertebrae. Care was taken when handling animals in order to avoid any unnecessary suffering. The abdominal cavity was incised and pups immediately removed, euthanised with chloroform, decapitated and entire heads placed immediately upon ice. Postnatal animals were injected intraperitoneally with a solution of sodium pentobarbital (180-200mg/ml)/euthanised with chloroform, prior to decapitation as per embryos. The brain was carefully dissected from the cranial cavity with the aid of microdissection tools and a dissecting microscope. All procedures were conducted according to local guidelines under ethical committee approval. Brains from embryonic C57BL/6J mice were additionally obtained through a collaboration with Dr. Graham Hagger and colleagues (GlaxoSmithKline Laboratories, Hertfordshire).

Some foetal and adult mouse brains were fixed with 4% paraformaldehyde solution, frozen in isopentane immersed in liquid nitrogen, and sectioned serially at 20-40μm using a cryostat. Surplus sections were stored in cryoprotectant (15% glucose, 30% ethylene glycol in PBS pH7.4: 75g sucrose, 150ml ethylene glycol made up to 500ml with PBS) at -20°C until further use. The majority of embryonic (2 litters at each time point from E15-birth), postnatal (P6, P12 and P19) and adult mouse brains were placed in either Bouin's fixative solution (made up as a solution containing 150ml saturated aqueous picric acid solution, 50ml of 40% formaldehyde solution, 10ml of glacial acetic acid) or neutral buffered formalin for 16-24 hours. Bouin's picric acid fluid is widely recognised as the fixative of choice in order to preserve the structural integrity of embryonic and foetal tissues. However, excess time in this fixative was avoided, as it led to marked hardening and shrinkage of the tissue. Picric acid is known to react with histones and basic proteins, forming crystalline picrates with amino acids. It stains tissue yellow, but this can be removed by placing specimens in 70% alcohol, or through treatment with lithium carbonate. The yellow colouration of the material was also useful for structural identification and orientation of samples in paraffin blocks following processing. Tissue from the spleen and lymph nodes of adult mice were also taken, to serve as positive control materials.

The processing schedule used routinely for human materials, was optimised for embryonic murine material, and was found to be equally suitable for tissues obtained from adult mice. Following fixation, tissues were processed in cassettes on a Shandon Hypercentre set to the

following schedule: (i) 50% alcohol for 30 minutes; (ii) 70% alcohol for 30 minutes: (iii) 96% alcohol for 30 minutes; (iv) 100% alcohol for 3 hours. (v) chloroform for 2 hours: (vi) paraffin wax, a mixture of dental wax and white wax, for 3 hours, prior to embedding into blocks and leaving to set on a cold plate. A total of 18 Bouin's-fixed foetal Balb/c and 36 C57BL/6J mouse brains (E15 to birth) were examined in this study. Each block of tissues contained foetal mouse brains from one litter at one time point. Blocks were presented with the rostral (olfactory) region to the uppermost (cutting) surface. Coronal blocks of tissue were sectioned serially at 15-40μm thickness on a base-sledge microtome, onto glass slides coated with aminopropyl silane for histological examination. Every tenth slide was selected for analysis, using immuno- and lectin histochemistry, essentially according to the protocols described for human tissues. The yellow colouration of Bouin's-fixed material was readily removed through the routine procedures of dewaxing and rehydration for sections on slides. Where necessary, a longer duration in 70% alcohol (a few hours or overnight, depending on thickness of sections) with continuous gentle agitation was sufficient to remove any residual yellow tincture from the sections. Adjacent slides were stained with haematoxylin and eosin for histological identification of brain areas. The list of reagents used is shown in **Table 10**. For immunohistochemistry, the secondary reagents used were biotinylated rabbit anti-rat IgG (1:50 dilution, mouse preadsorbed, Vector Laboratories, UK), rabbit anti-goat IgG (1:100 dilution, Vector laboratories, UK) or swine anti-rabbit IgG (1:100 dilution, Dako, UK). Sections were incubated with appropriate corresponding normal sera (rabbit or swine) for blocking steps prior to incubation with the primary antibody, as per protocol in Chapter 2. Slides were counterstained lightly with haematoxylin or with methyl green.

Table 10. Antibodies used for immunohistochemistry to rodent determinants, and lectins for histochemistry

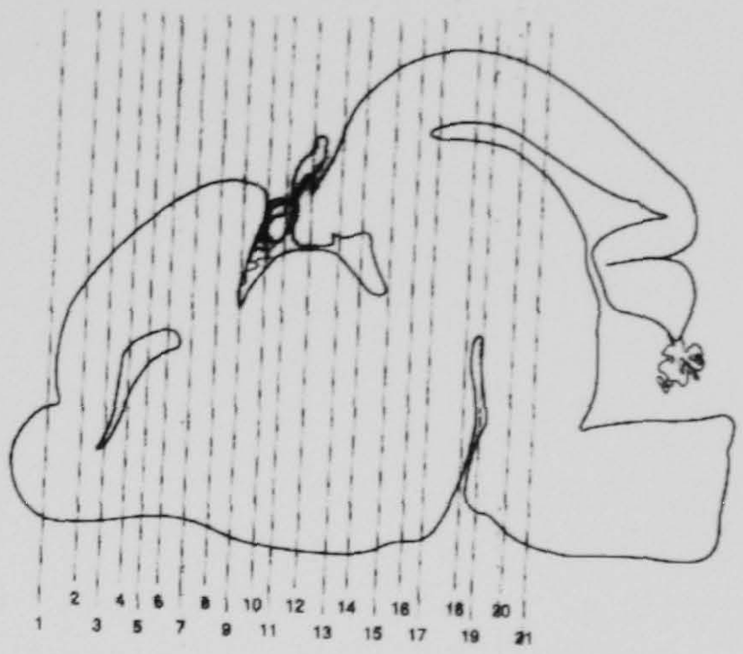
	Clone	Presentation	Dilution	Source
Antibodies to mononuclear phagocytes/leukocytes				
CD11b (rat anti-mouse Mac-1/Ly40)	MI/70.15.1	IgG_{2b}	1:10	Serotec, Ltd., UK
CD45 (rat anti-mouse LCA/Ly5)	SRT5	IgM	1:10	Serotec Ltd., UK
CD45 (rat anti-mouse)	YW 62.3	IgG_{2b}	1:10	Serotec Ltd., UK
F4/80 (rat anti-mouse)	Cl: A3-1	IgG_{2b}	1:10	Serotec Ltd., UK
Metallophilic macrophages (rat anti-mouse)	MOMA-1	IgG_{2c}	1:10	Serotec Ltd., UK
Antibodies to adhesion molecules				
CD31 (rat anti-mouse PECAM-1)	MEC 13.3	IgG_{2a}	1:10-1000	Cambridge Bioscience, UK
CD54 (rat anti-mouse ICAM-1)	KAT-1	IgG_{2a}	1:10-1000	Serotec Ltd., UK
CD102 (rat anti-mouse ICAM-2)	3C4 (MIC2/4)	IgG_{2a}	1:10-1000	Cambridge Bioscience, UK
CD106 (rat anti-mouse VCAM-1)	M/K-2	$IgG_{1\kappa}$	1:10-1000	Serotec Ltd., UK
CD106 (rat anti-mouse VCAM-1)	429	IgG_{2a}	1:10-1000	Canbridge Bioscience, UK
Antibodies to cytokines				
MIP-1α (goat anti-mouse)	---	IgG	1:10-1000	R&D Systems, UK
MCP-1 (hamster anti-mouse)	2H5	IgG	1:10-1000	Cambridge Bioscience, UK
MCP-1 (rabbit anti-rat/mouse)	---	IgG	1:100	Serotec Ltd., UK
GMCSF (rabbit anti-mouse)	---	IgG	1:10-1000	Serotec Ltd., UK
Biotinylated lectins				
Ricinus communis agglutinin-1 [RCA-1/RCA_{120}]	---	---	1:250-500	Vector Labs., UK
Griffonia bandeiraea simplicifolia isolectin B4 [GSB4]	---	---	1:100-500	Vector Labs., UK
Lycopersicon esculentum [Tomato lectin]	---	---	1:100-250	Vector Labs., UK

Results

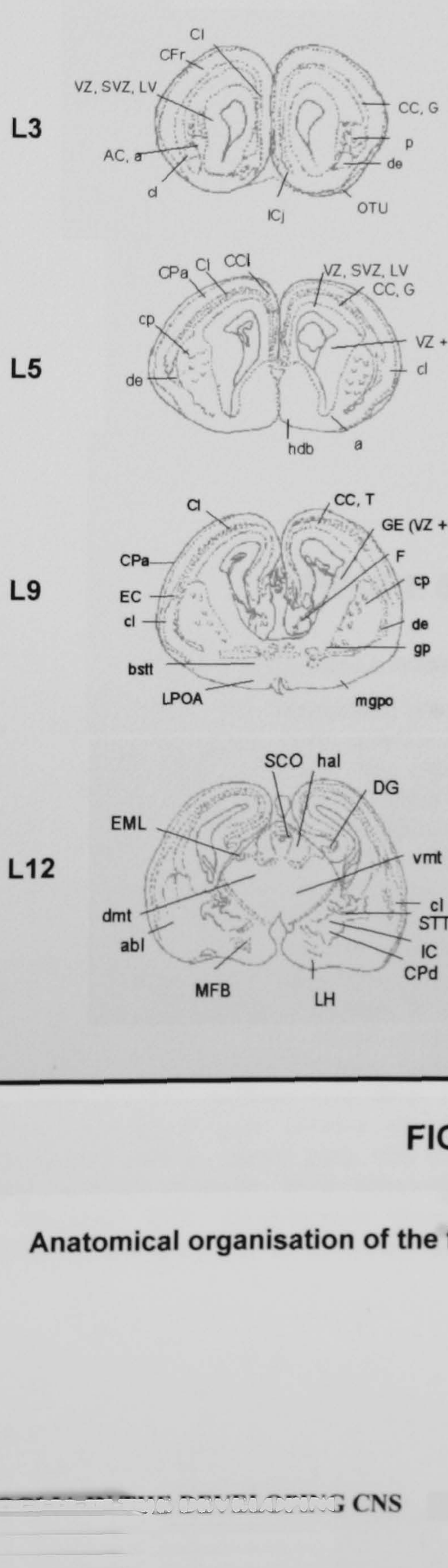
Distribution, morphology and phenotype of microglial progenitors in foetal murine CNS

Schematic, annotated outlines of coronal sections from the developing brain are indicated in **Figure 62** and **Figure 63**, to show the regional anatomy of the developing mouse brain. These were compiled with reference to standard atlases (Jacobowitz et al. 1997; Schambra et al. 1992). Section levels indicated on the figure progress in a rostral-caudal direction. The regional distributions of tomato lectin and RCA-1 positive amoeboid and early ramifying microglia in the embryonic mouse brains are shown in **Figure 64** and **Figure 65** for comparison. Outlined sections correspond approximately to the section levels indicated in **Figure 62** and **Figure 63**. Schematic plots represent cumulative data from three sections analysed per embryonic brain, at each level, per time point. Individual identified cells were initially plotted manually onto draft templates of the brain sections, prior to being collated and presented in the final digitised format. Amoeboid microglia were defined as morphologically amorphous or rounded cells with a defined nucleus (stained with haematoxylin). Early ramifying cells possessed a small nucleus and two or more elongating and delicate processes. Only cells that could conclusively be identified were used for plotting data. There were no clear strain differences between C57BL/6J or Balb/c mice in the distribution patterns of microglia during the embryonic period. In order to facilitate regional comparison between the distribution of the two morphological varieties, the left hemisphere was used to denote amoeboid microglia (blue), and the right hemisphere for early ramified cells (yellow). At all stages examined, amoeboid cells appeared to coexist regionally with progressively ramifying cells, with the exception that these early ramified microglia appeared to migrate and colonise the grey matter with ongoing development, whereas amoeboid cells tended to predominate within developing (white matter) tracts.

The mononuclear phagocyte markers, CD11b, CD45 and MOMA could not be identified on microglial progenitors, amoeboid cells or early ramifying throughout the embryonic period. These markers produced specific immunoreactivity within control tissues (spleen and lymph nodes). Only F4/80 antigen was found to be expressed to variable degrees on some populations of microglia during the period under study. GSB4 lectin was far less useful as a marker for microglia in the developing brain, than either tomato lectin or RCA-1, which reliably detected the majority of microglial forms. For this reason, the data accrued from analysis of sections reacted with these two lectins were used for the comparative representations shown in **Figure 64** and **Figure 65**.



E15 Coronal Section Levels



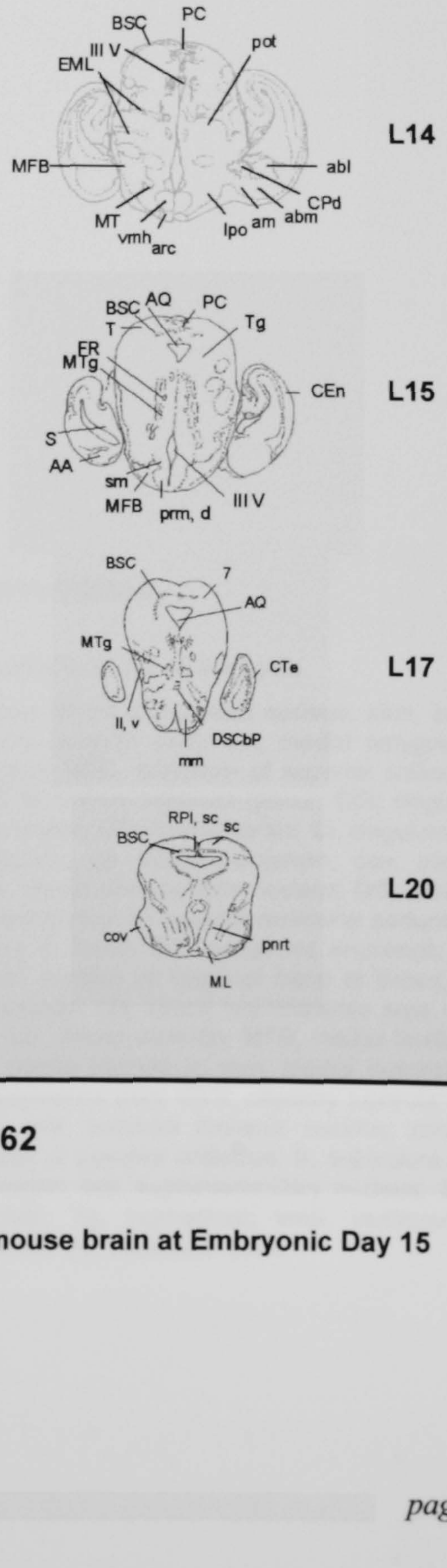


FIGURE 62

Anatomical organisation of the foetal mouse brain at Embryonic Day 15

FIGURE 62: *abbreviations*

Corresponding section levels are indicated (L1-L21) on the figure.

a, accumbens nucleus; **AA**, amygdaloid area; **abl**, basal lateral amygdaloid nucleus; **abm**, basal medial amygdaloid nucleus; **Aca**, anterior commissure (anterior part); **am**, medial amygdaloid nucleus; **AQ**, aqueduct of Sylvius; **arc**, arcuate nucleus; **BSC**, brachium of superior colliculus; **bsttl**, bed nucleus of stria terminalis (lateral part); **CC G**, corpus callosum (genu); **CCi**, cingulate cortex; **CC T**, corpus callosum (trunk); **CEn**, entorhinal cortex; **CFr**, frontal cortex; **Cl**, cingulum; **cl**, claustrum; **CPa**, parietal cortex; **CPd**, cerebral peduncle; **cp**, caudate-putamen; **cov**, ventral cochlear nucleus; **CTe**, temporal (auditory) cortex; **de**, dorsal endopyriform nucleus; **DG**, dentate gyrus; **dmt**, dorso-medial thalamic nucleus; **DSCbP**, decussation of superior cerebellar peduncles; **EC**, external capsule; **EML**, external medullary lamina; **F**, fornix; **GE**, ganglionic eminence; **gp**, globus pallidus; **hal**, lateral habenula; **hdb**, horizontal nucleus of diagonal band of Broca; **IC**, internal capsule; **ICj**, islands of Calleja; **III V**, third ventricle; **LH**, lateral hypothalamic area; **lpo**, lateral preoptic nucleus; **LPOA**, lateral preoptic area; **LV**, lateral ventricle; **MFB**, medial forebrain bundle; **mgpo**, magnocellular preoptic nucleus; **ML**, medial lemniscus; **mm**, medial mammillary nucleus; **MT**, mammillothalamic tract; **MTg**, mammillotegmental tract; **OTU**, olfactory tubercle; **PC**, posterior commissure; **pnrt**, pontine reticular nuclei; **pot**, posterior thalamic nucleus; **prm d**, premammillary nucleus (dorsal part); **RPI sc**, roof plate of superior colliculus; **S**, subiculum; **sc**, superior (anterior) colliculus; **SCO**, subcommissural organ; **sm**, supramammillary nucleus; **STT**, stria terminalis; **SVZ**, subventricular zone; **T**, tectum; **Tg**, tegmentum; **vmh**, ventromedial hypothalamic nucleus; **vmt**, ventromedial thalamic nucleus; **VZ**, ventricular zone.

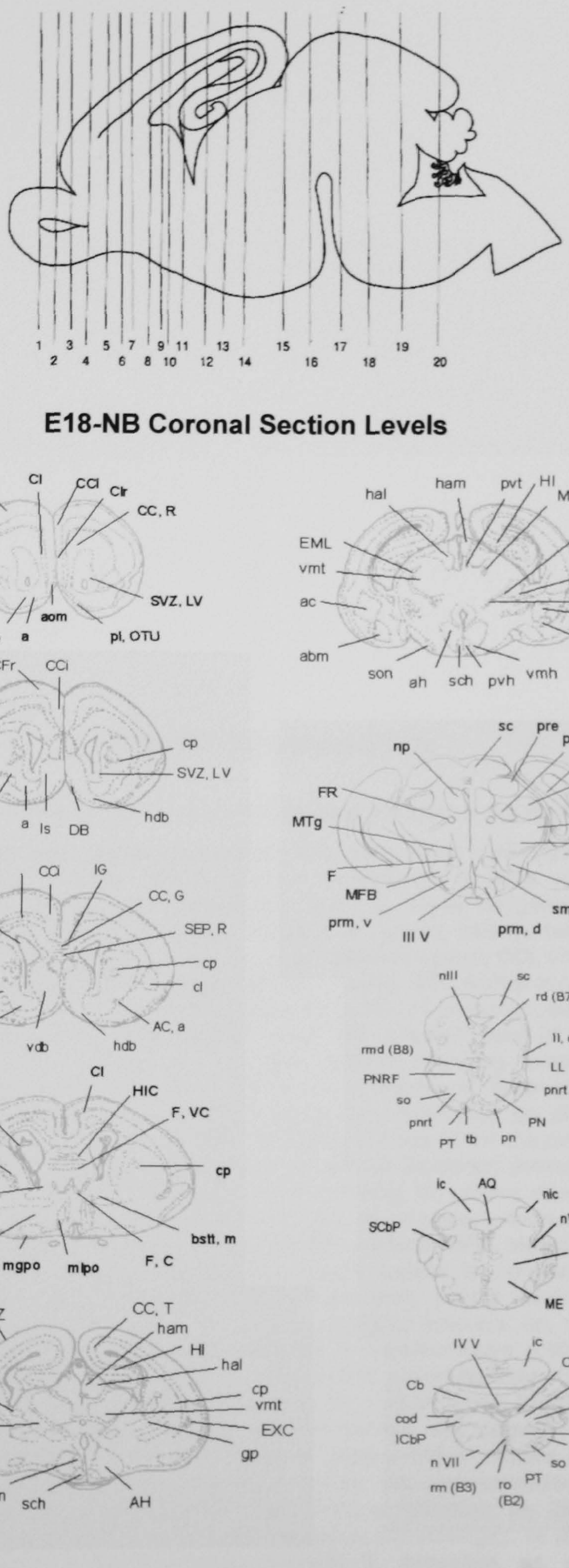


FIGURE 63

Anatomical organisation of the foetal mouse brain E18-Birth (NB)

FIGURE 63: *abbreviations*

Corresponding section levels are indicated (L1-L20) on the figure.

a, nucleus accumbens; **AAA**, anterior amygdaloid area; **abm**, basal medial amygdaloid nucleus; **ac**, central amygdaloid nucleus; **AC a**, anterior commissure (anterior part); **ah**, anterior hypothalamic nucleus; **AH**, anterior hypothalamic area; **al**, lateral amygdaloid nucleus; **aom**, anterior olfactory nucleus (medial part); **AQ**, aqueduct of Sylvius; **bstt m**, bed nucleus of stria terminalis (medial part); **Cb**, cerebellum; **CC G**, corpus callosum (genu); **CCi**, cingulate cortex; **CC R**, corpus callosum (rostrum); **CC T**, corpus callosum (trunk); **CFr**, frontal cortex; **ChPl**, choroid plexus; **Cl**, cingulum; **CIn**, insular cortex; **CIr**, infralimbic cortex; **cl**, claustrum; **cod**, dorsal cochlear nucleus; **cp**, caudate-putamen; **CPa**, parietal cortex; **DB**, diagonal band of Broca; **de**, dorsal endopyriform nucleus; **EC**, external capsule; **EML**, external medullary lamina; **EXC**, extreme capsule; **F**, fornix; **F C**, column of the fornix; **FR**, fasciculus retroflexus (habenula-peducular tract); **F VC**, ventral commissure of the fornix (ventral hippocampal commissure); **gp**, globus pallidus; **hal**, lateral habenula; **ham**, medial habenula; **hdb**, horizontal nucleus of the diagonal band of Broca; **HI**, hippocampus; **HIC**, hippocampal commissure; **ic**, inferior (posterior) colliculus; **ICbP**, inferior cerebellar peduncle; **III V**, third ventricle; **IV V**, fourth ventricle; **IC**, internal capsule; **IG**, indusium griseum; **II d**, lateral lemniscus nucleus (dorsal part); **lpo**, lateral preoptic nucleus; **ls**, lateral septal nucleus; **LL**, lateral lemniscus; **LV**, lateral ventricle; **ME**, medulla; **MFB**, medial forebrain bundle; **mg**, medial geniculate body; **mgpo**, magnocellular preoptic nucleus; **ML**, medial lemniscus; **mlpo**, medial preoptic nucleus; **MTg**, mammillotegmental tract; **mvb**, medial vestibular nucleus; **MZ**, marginal zone; **n III**, oculomotor nucleus; **nic**, nucleus of inferior colliculus; **np**, nucleus proprius of posterior commissure; **nVmes**, mesencephalic nucleus of trigeminal nerve; **n VII**, nucleus of facial nerve; **nVsp**, spinal tract nucleus of trigeminal nerve; **pl CPf**, pyramidal layer of pyriform cortex; **pl OTU**, pyramidal layer of olfactory tubercle; **PN**, pons; **pn**, pontine nuclei; PNRF, pontine reticular formation; **pnrt**, pontine reticular nuclei; **pre**, pretectal nucleus; **pot**, posterior thalamic nucleus; **prm d**, premammillary nucleus (dorsal part); **prm v**, premammillary nucleus (ventral part); **PT**, pyramidal tract; **pvh**, paraventricular hypothalamic nucleus; **pvt**, paraventricular thalamic nucleus; **rd (B7)**, dorsal raphe nucleus (serotonergic); **ReF**, reticular formation; **rm** (B3), nucleus raphe magnus (serotonergic); **rmd** (B8), nucleus raphe medianus (serotonergic); **ro** (B2), nucleus raphe obscurus (srotonergic); **rt**, reticular thalamic nucleus; **S**, subiculum; **sc**, superior (anterior) colliculus; **SCbP**, superior cerebellar peduncle; **sch**, suprachiasmatic nucleus; **sdn**, sexual dimorphic nucleus; **SEP R**, septum pellucidum (roof); **sm**, supramammillary nucleus; **so**, superior olivary nucleus; **sol**, solitary tract nucleus; **son**, supraoptic nucleus; **STT**, stria terminalis; **SUT**, subthalamus; **SVZ**, subventricular zone; **tb**, nucleus of trapezoid body; **vdb**, ventral nucleus of diagonal band of Broca; **vlg**, ventral lateral geniculate body; **vmh**, ventromedial hypothalamic nucleus; **vmt**, ventromedial thalamic nucleus; **VP**, ventral pallidum; **zi**, zona incerta.

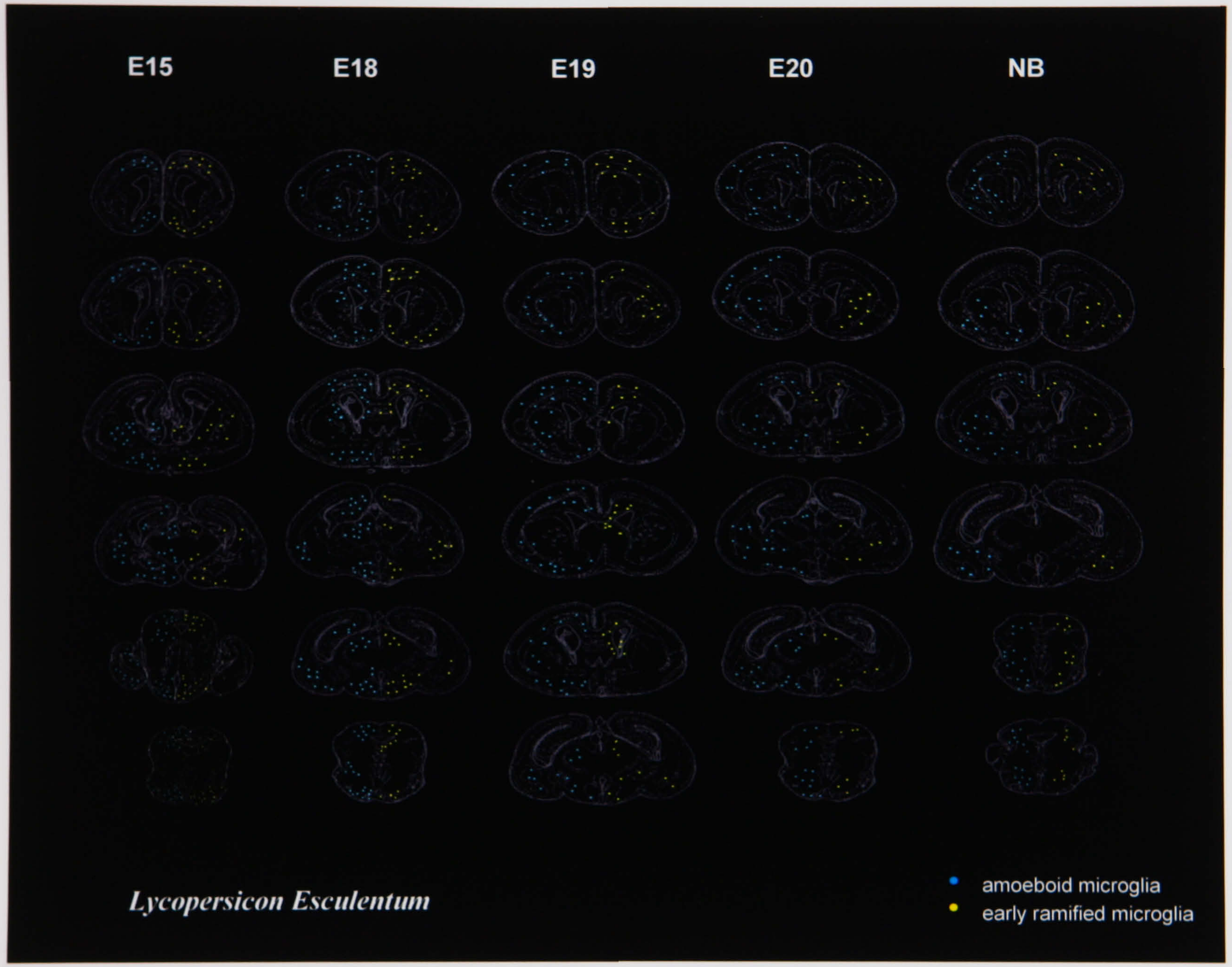


FIGURE 64

Regional distribution of tomato lectin-positive microglia in the developing mouse brain from E15 to birth

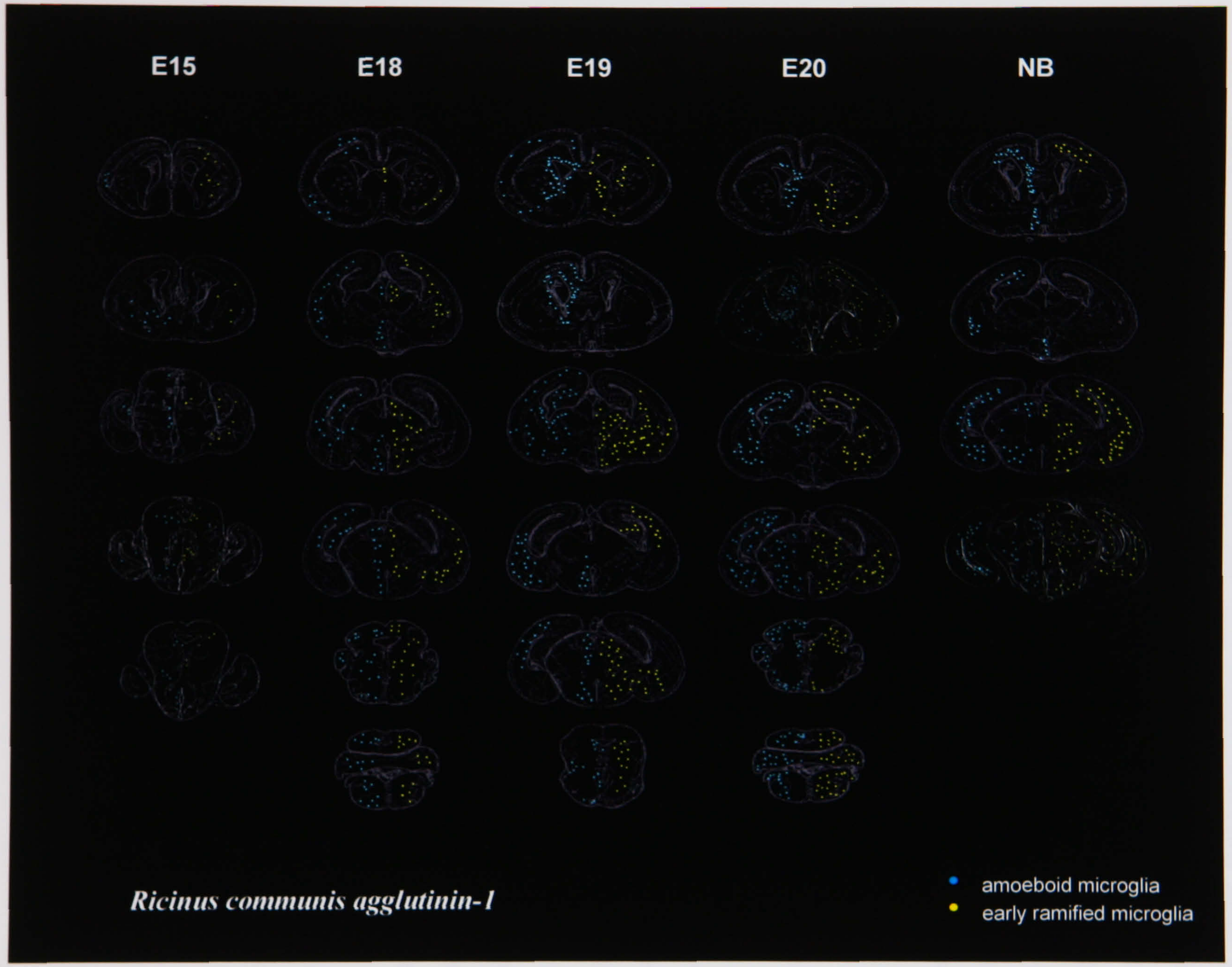


FIGURE 65

Regional distribution of RCA-1 lectin-positive microglia in the developing mouse brain from E15 to birth

Tomato lectin clearly identified microglial progenitors and amoeboid cells at E15, located mainly at perivascular sites and associated with infolding of the meninges (**Figure 66**). These cells were distributed in dorsal and ventral regions of the brain and particularly dense around the aqueduct, in the tectum, tegmentum and around the pontine nuclei (**Figure 64**). Microglial progenitors identified between E15 and E16 were closely apposed to the parenchymal wall of cerebral blood vessels or positioned at short distances away from vessels (**Figure 66 F-K**). The majority were found within forebrain ventricular and subventricular zones, in the corpus callosum, internal capsule, preoptic area, within areas bordering the caudate-putamen, as well as within the tectum (superior colliculus), tegmentum and pons. Very rarely were progenitors identified within blood vessels. Amoeboid microglia were typically found in transitory white matter tracts (corpus callosum and internal capsule) at some distance from blood vessels (**Figure 66 L-N**). Foetal microglia had already begun to differentiate into bipolar and tripolar forms (**Figure 66 O-Q**), progressing to multipolar cells with long and delicate processes. The majority of these cells maintained their close relationship with blood vessels.

The cerebrovasculature had clearly advanced by E17, forming distinguishable networks particularly following caudal to rostral and ventral to dorsal gradients (in such areas as the medulla- **Figure 67**) and the ventral diencephalon. Within these highly vascularised regions, microglial differentiation was also more at E17 (**Figure 67**). Amoeboid cells anddifferentiating microglia still prevailed within the VZ and SVZ. By E18, amoeboid and a few ramifying cells could be found within the hippocampus and dorsal thalamus (**Figure 68**), within the amygdaloid area, hypothalamus, around the aqueduct and basal pons. Microglial progenitors and ramified cells could be seen frequently at perivascular sites. Labelled progenitors could occasionally be identified within vessels passing through the ventricular zone (**Figure 68D**). Amoeboid and ramifying microglia were further identified within grey matter nuclei (caudate and thalamus) as well as in neural tracts. The ramifying variety were increasingly difficult to identify using tomato lectin, probably on account of a lower level expression of surface (N-acetylglucosamine containing) glycoconjugates with progressive morphological transformation (**Figure 68H,I**).

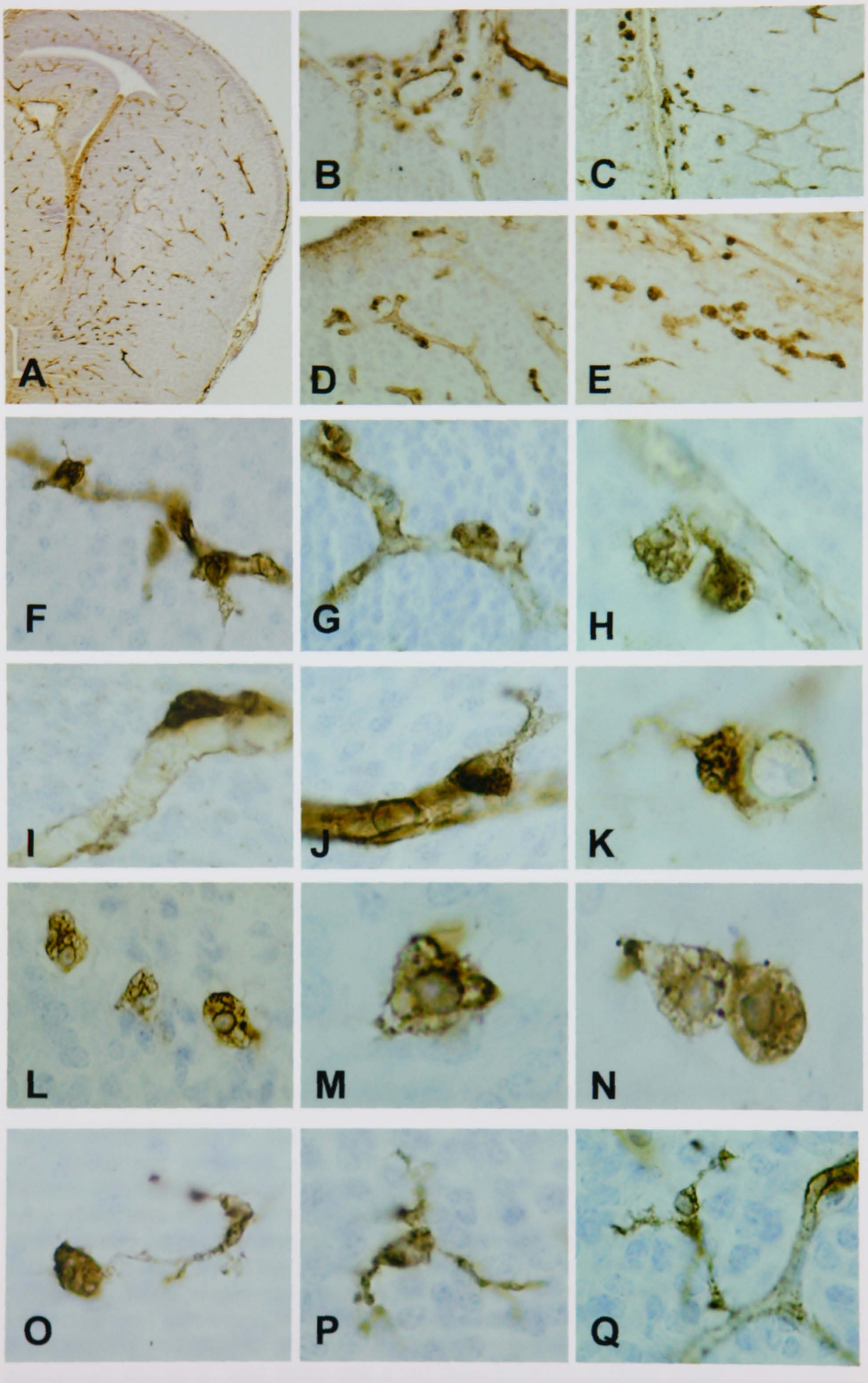


FIGURE 66

Tomato lectin-positive microglia in the mouse brain at E15

FIGURE 66 *(continued)*

Tomato lectin-positive microglia in the mouse brain at E15

Tomato lectin positive vessels and foetal microglia were detected throughout the brain (**A**). Round and amoeboid cells were found around meningeal vessels (**B**), at the juncture between meningeal infolding and the CNS parenchyma (**C**), and a few cells were associated with blood vessels in the ventricular and subventricular zones (**D,E**). Blood vessel-associated progenitors were frequently detected, most of these in the vicinity of, or adherent to the parenchymal wall of blood vessels (**F,G**). The majority of these early blood vessel-associated progenitors (**H-K**) were identified in the forebrain ventricular and subventricular zones, corpus callosum and at the boundary between these regions and caudate-putamen, the internal capsule, in the preoptic area, tectum, tegmentum and pons. Very rarely was a cell detected within a blood vessel (not shown). Amoeboid cells at some distance from vessels (**L-N**) could be seen in the corpus callosum (and cingulate cortex) and its extensions, as well as within the internal and external capsules bordering the caudate-putamen. A small number of microglia had already begun to differentiate at this age to cells with bipolar and tripolar morphologies (**O-Q**). Many of these differentiating cells maintained their close relationship with blood vessels (**Q**).

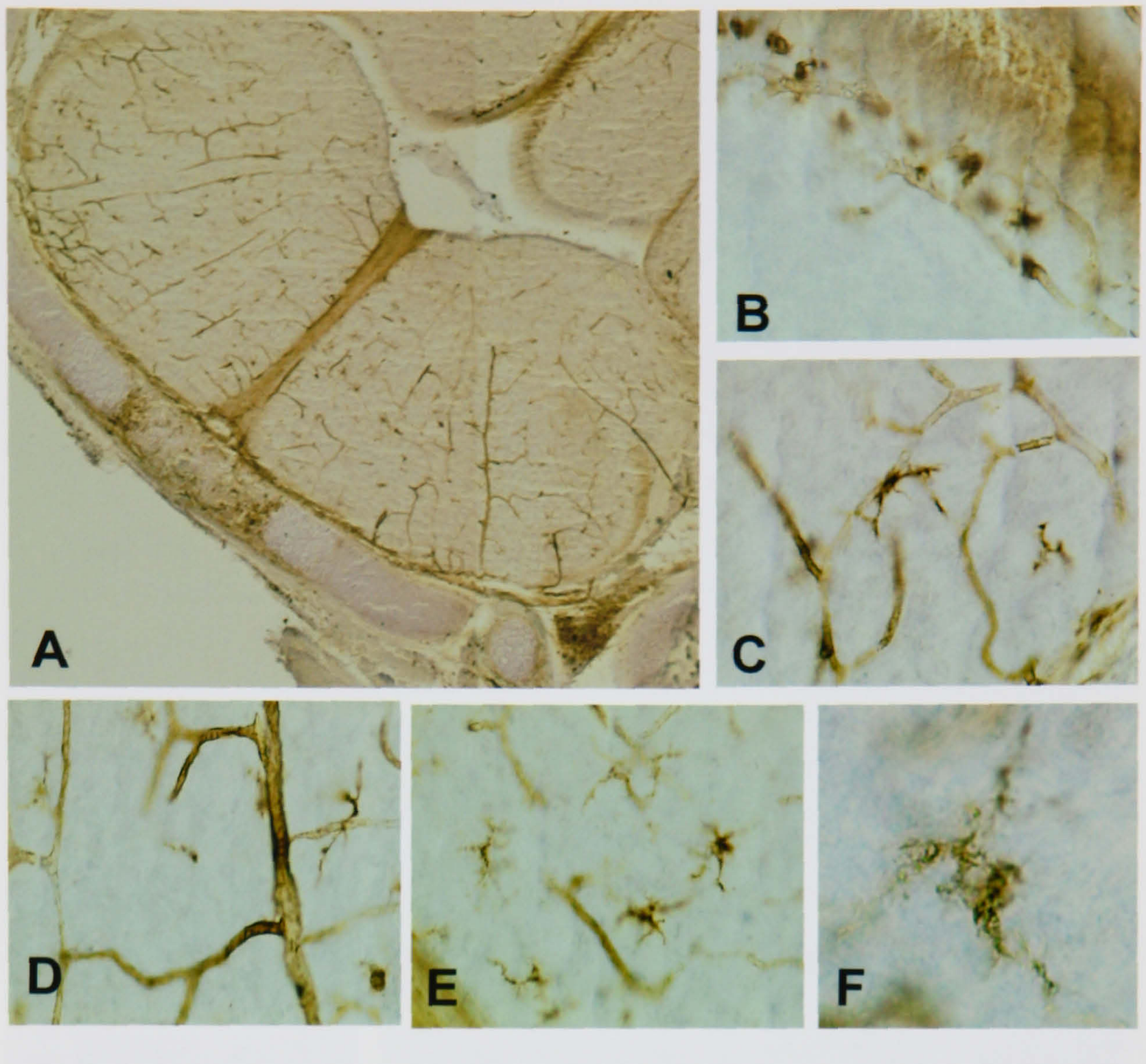


FIGURE 67

Tomato lectin-positive early ramified microglia in the mouse brain at E17

Regional development of the cerebral vasculature had clearly advanced (particularly in caudal-rostral and ventral to dorsal gradients) by E17, with blood vessels penetrating structures such as the medulla (**A**) and the ventral diencephalon (not shown). With the establishment of the vascular network in these regions, microglia clearly also began to differentiate and adopt more mature, ramified forms (**C-F**). Amoeboid cells and early ramified cells continued to predominate within the ventricular (**B**) and subventricular zones.

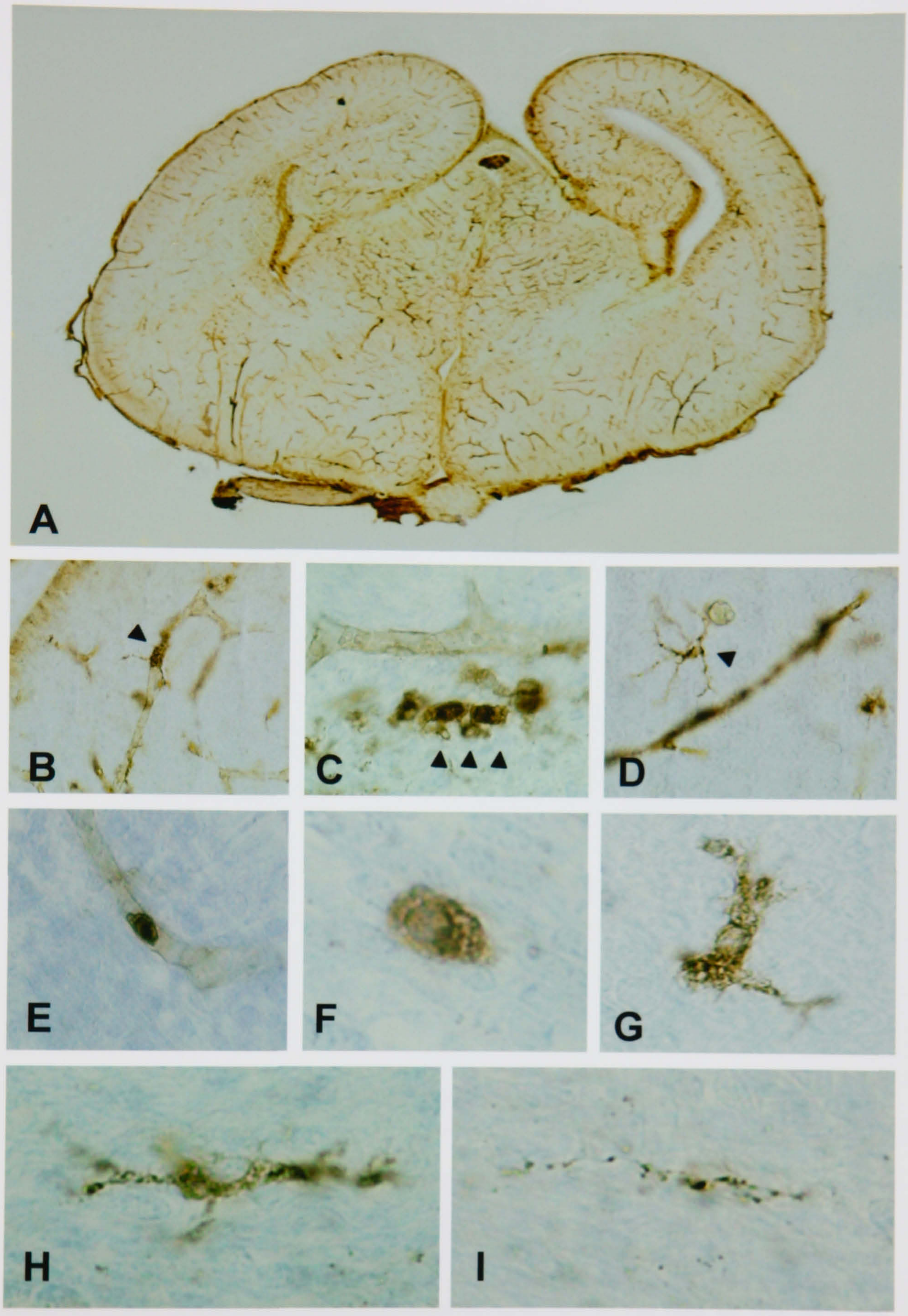


FIGURE 68

Tomato lectin-positive microglia in the mouse brain at E18

FIGURE 68 *(continued)*

Tomato lectin-positive microglia in the mouse brain at E18

A section of the mouse brain at E18, histochemically reacted with tomato lectin and showing the thalamus and hippocampus, is indicated in (**A**). This corresponds approximately to section level 13 on Figure 63. Amoeboid and some few ramifying cells could be found in the hippocampus and dorsal thalamus at this age, as well as in the amygdaloid area, hypothalamus, around the aqueduct of Sylvius and basal pons. These areas were highly vascularised. Microglial progenitors were predominantly located at perivascular sites (**B-D**). Occasionally, labelled progenitors could be seen passing through vessels in ventricular zones (**E**). Amoeboid cells (**F**) and cells with more ramified bipolar and tripolar cell morphologies (**G-I**) could be identified within grey matter nuclei (caudate, thalamus) and within white matter tracts. However, these latter variety became more difficult to detect using tomato lectin (perhaps on account of downregulating surface carbohydrate residues (glycoconjugates containing N-acetylglucosmanine) recognised by this lectin (**H,I**) in the process of differentiation.

In contrast to the progressively fewer microglia detected using tomato lectin, RCA-1 lectin histochemistry continued to identify the ramifying population (in addition to the amoeboid microglia) (**Figure 65**). This may suggest that whereas microglia downregulate certain glycoconjugates (e.g. those containing N-acet ylglucosamine) with advancing development *in utero*, others (e.g. those containing lactose and galactose) are maintained or even upregulated on differentiated cells. The discrepancy between lectin-positive microglial distribution was clearly evident from E19 onwards (**Figure 64 and Figure 65**). This finding may have some relevance related to the function of microglia, for example in adherence to ECM components or in their interaction with other cells in the CNS.

At E19, tomato lectin clearly identified a surge of newly-emergent progenitors and amoeboid cells within the cavum septum pellucidum (**Figure 69**). These cells appeared larger than the progenitors identified around vessels at E15, and were found clustered densely within the ventricular zone close to the rostral horn of the lateral ventricle, where they were associated with thin-walled blood vessels. A small proportion were dispersed in the overlying corpus callosum and a few cells had wandered out onto the ependymal surface lining the ventricles. Amoeboid cells were particularly notable within the cavum septum pellucidum at E20 (**Figure 70**), and now formed a continuous band of cells subjacent to the corpus callosum. Large amoeboid cells tended to accumulate within periventricular regions and there were some indications that these cells were extravasating within these areas (**Figure 70**). In newborn animals, ramified RCA-1 positive cells could be clearly seen in highly vascularised sites, associated with well-differentiated vessels as shown in **Figure 71**. Far fewer ramified cells could be identified with tomato lectin in newborn animals. In the postnatal animals examined, amoeboid microglia were found to predominate within the corpus callosum at postnatal day 6 (P6) (**Figure 72 A-C**), while ramified cells were already present within the grey matter at this age (**Figure 72D**). The latter variety became progressively more differentiated and had downregulated their lectin-binding sites by P19 (**Figure 73**). As mentioned previously, GSB4 lectin was not suitable for detecting microglia in the murine CNS according to the protocol established in this study for tomato lectin and RCA-1 (**Figure 73**).

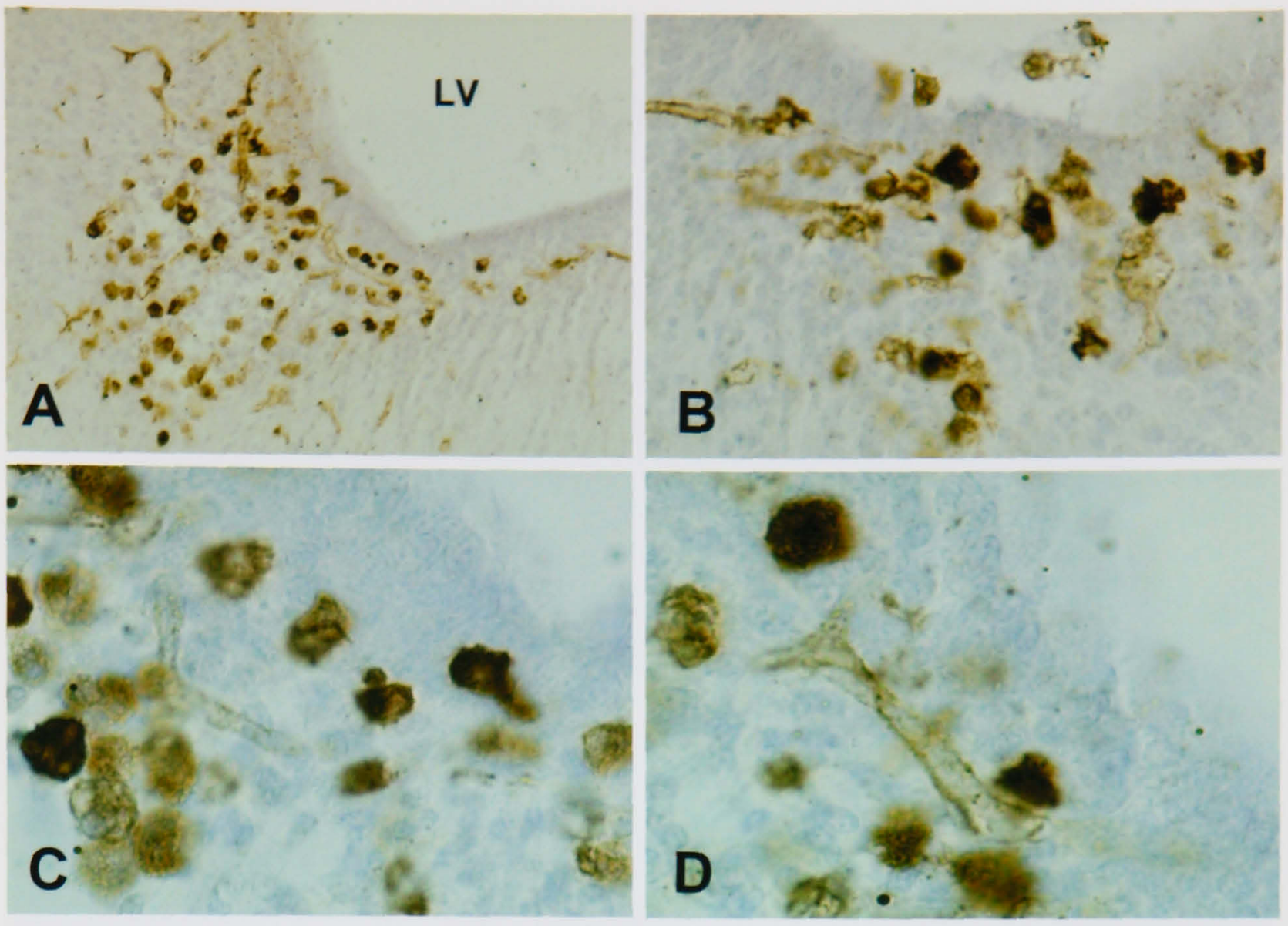


FIGURE 69

Tomato lectin-positive amoeboid cells in the cavum septum pellucidum of mouse brain at E19

At E19, a conspicuous population of lectin-positive cells emerged distinctly within the transitory cavum septum pellucidum (corresponding to section level 8) between the two rostral horns of the lateral ventricle (LV) (**A-D**). These cells were particularly dense within the ventricular zone immediately adjacent to the lateral ventricle (**C,D**), and very closely associated with small delicate vessels within these areas. They were larger than the microglial progenitors observed around E15, and resembled round macrophages more closely. In view of their relationship with the thin-walled capillaries, they appeared to have recently emigrated into the cavum from the bloodstream. The majority of cells dispersed into the cavum septum pellucidum and were occasionally detected in the overlying corpus callosum, while a small number could be seen attached to the ventricular surface of the ependymal lining and within the lateral ventricle (not shown), suggesting that they had migrated into the ventricle away from their original site of entry.

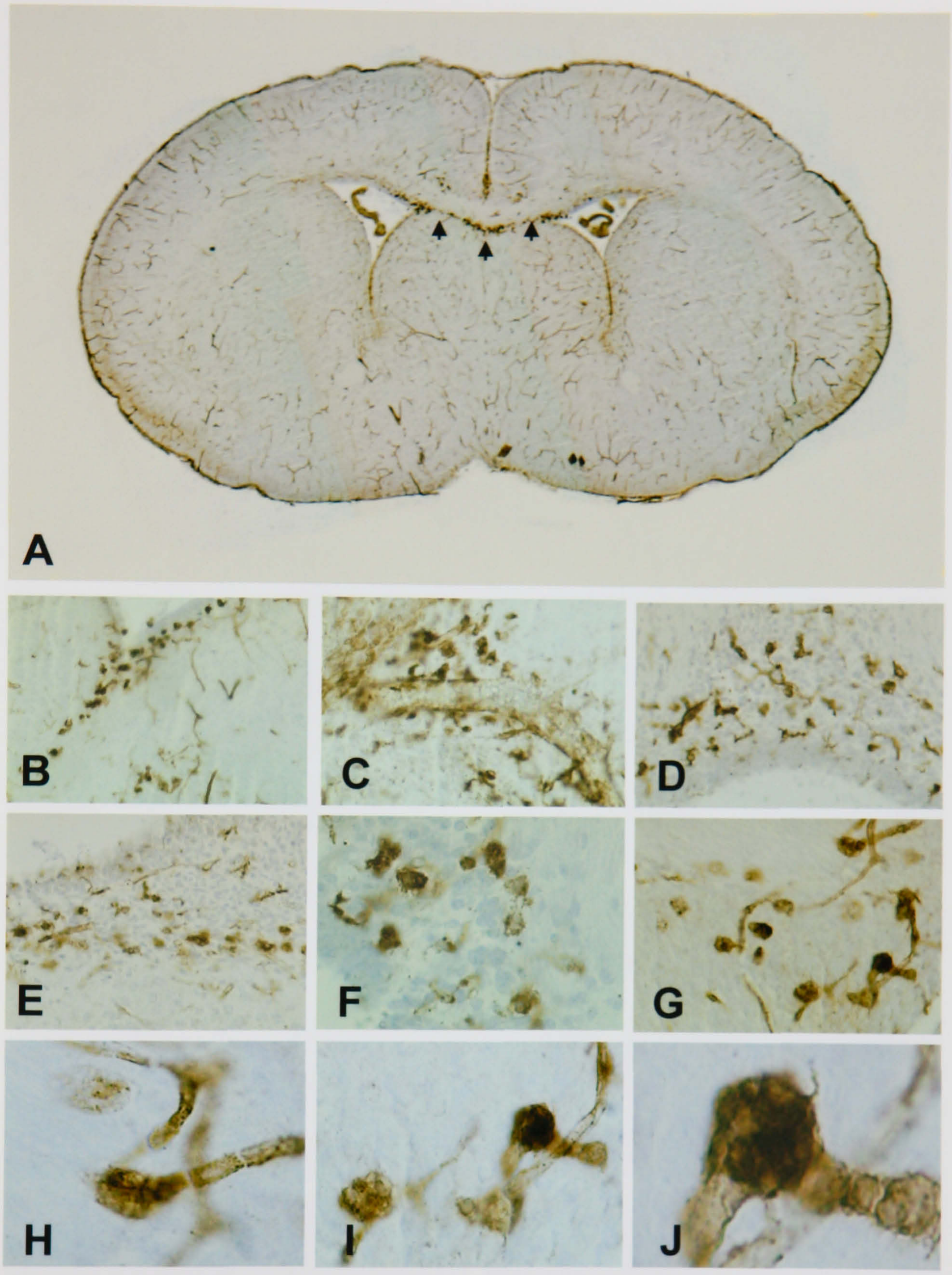


FIGURE 70

Tomato lectin-positive amoeboid cells in the cavum septum pellucidum and subjacent to the corpus callosum in the mouse brain at E20

At E20, amoeboid cells within the cavum septum pellucidum had migrated along the extent of this structure in a linear formation, underlying the corpus callosum (**A, arrowheads**). Blood vessels within this region and the ventricular zone bordering the head of the lateral ventricle, had also developed and become more distinguishable (**C,G**). These large amoeboid cells still tended to accumulate at periventricular sites (**B,D,E,F**), and they appeared to be extravasating within these regions (**H-J**).

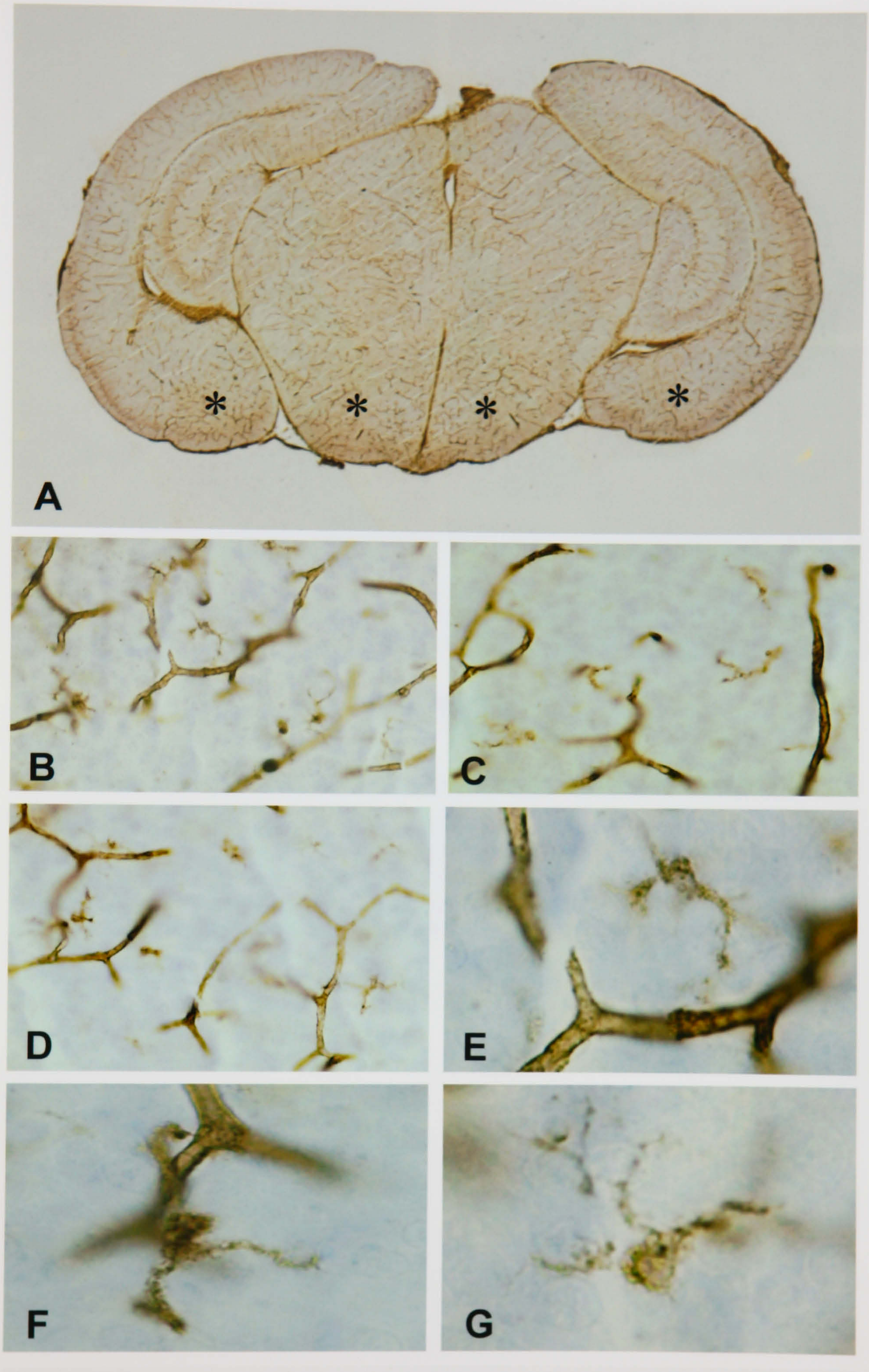


FIGURE 71

Lectin-positive microglia and blood vessels in the mouse brain at birth

In newborn animals, ramifying RCA-1 lectin positive microglia (**B-G**) were detected at highly vascularised sites within the CNS (**A, asterisks**), particularly associated with well-differentiated vessels in the ventral aspect of the brain. The section shown in (A) corresponds approximately to section level 14.

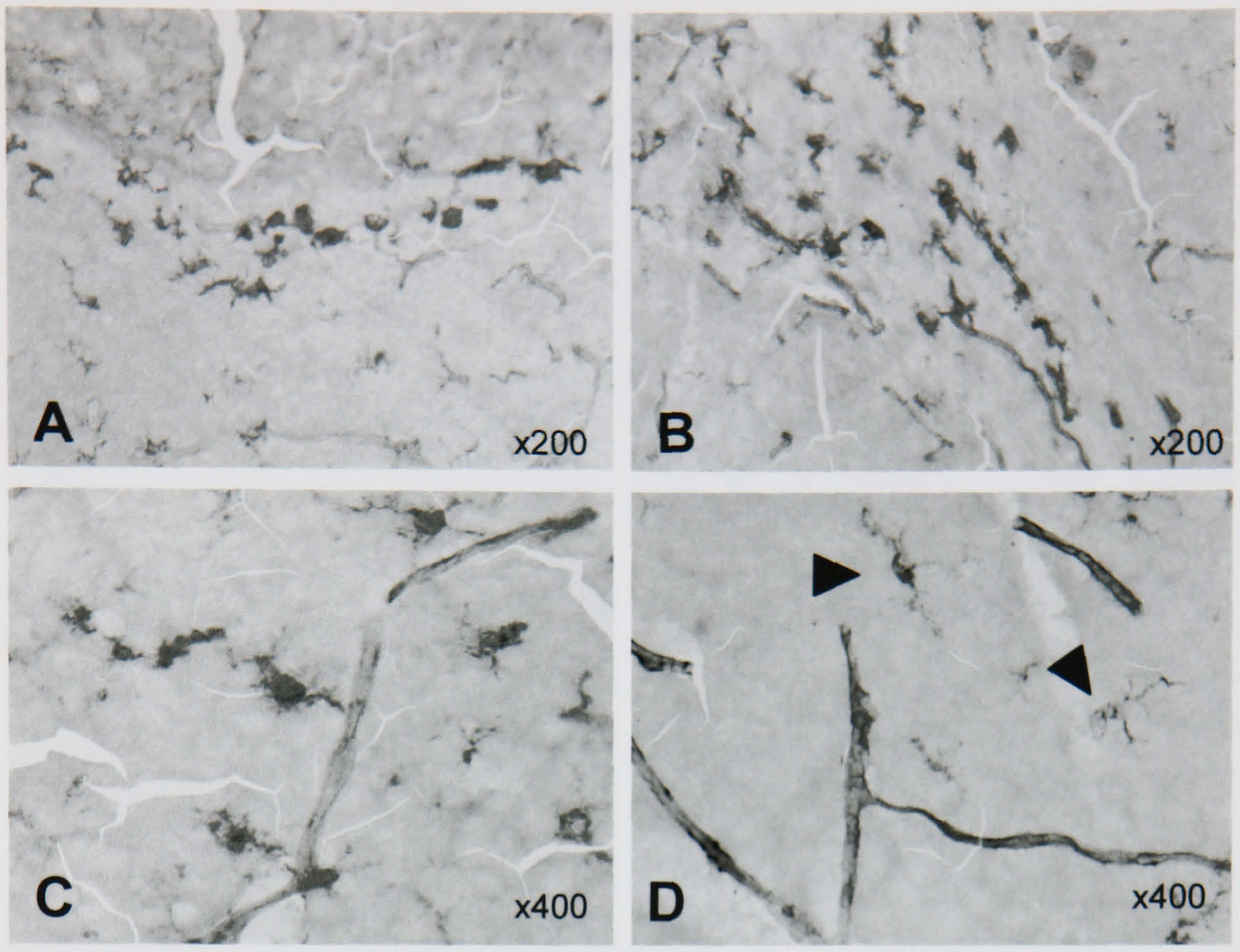


FIGURE 72

RCA-1 positive microglia and blood vessels in the mouse brain at P6

Six days following birth, amoeboid cells were seen within the corpus callosum (**A-C**) whereas ramified forms were located away from these major foci of accumulation. Within the grey matter (**D**), resting microglia which were in a far advanced state of differentiation were much fainter and more easily prone to being overlooked (**D, arrowheads**), by comparison to the amoeboid cells in the corpus callosum.

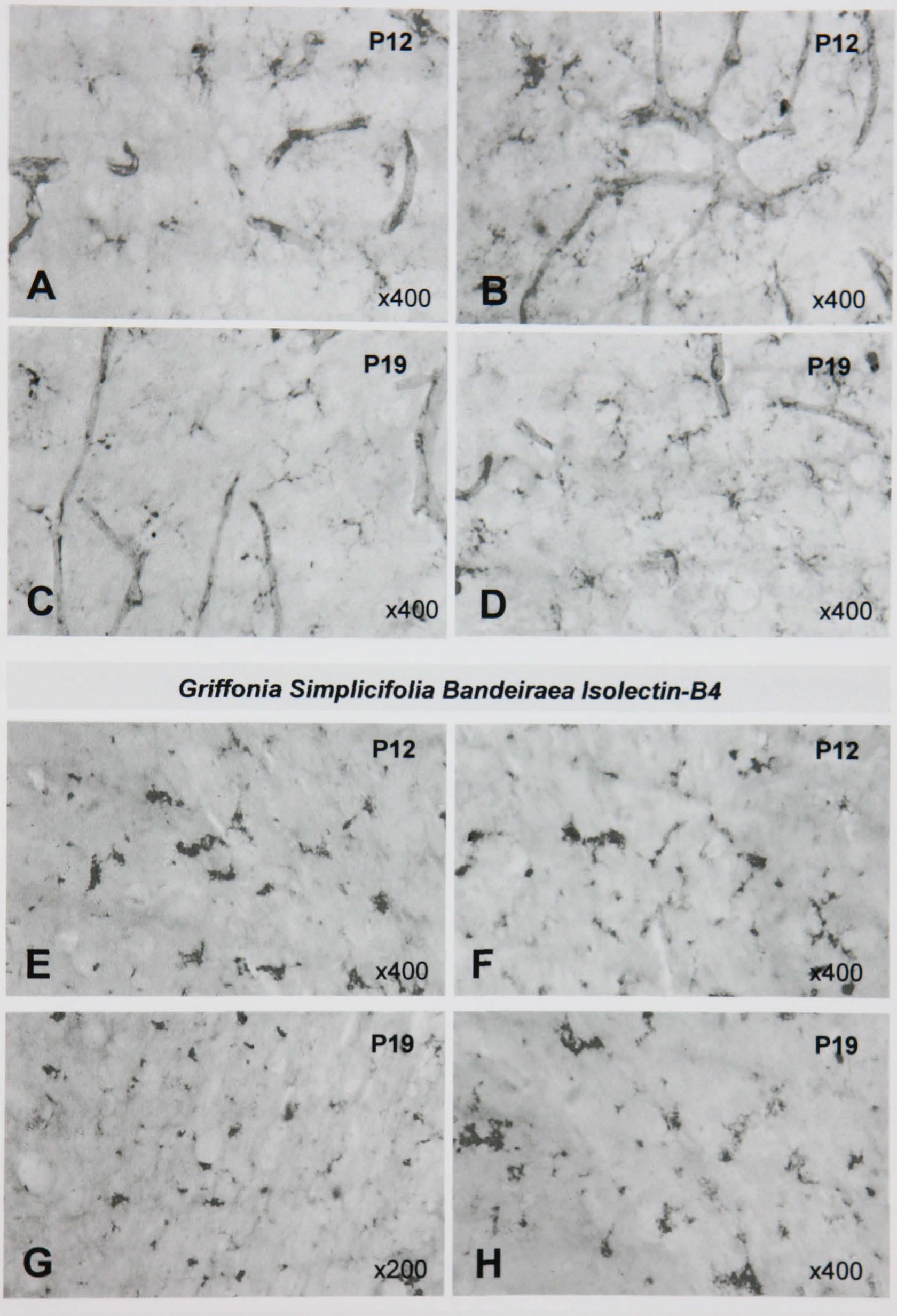


FIGURE 73

RCA-1 and GSB-4 positive histochemistry in the mouse brain at P12 and P19

Between the 12th and 19th postnatal days, parenchymal microglia differentiated further and were only weakly labelled with the RCA-1 lectin (**A-D**). This lectin continued to detect mature ramified cells at P19 (**C-D**). By contrast, GSB-4 was not found to be suitable for studying the differentiation of microglia using the procedure outlined for tomato lectin and RCA-1. As shown in the figure (**E-H**), GSB4 formed precipitates on the tissue section, thus masking any morphological characteristics that could be ascribed to microglia.

The results presented here have shown a topographical transition in morphology from amoeboid to ramified forms of microglia, that takes place around E17-E19 and beyond, based on lectin histochemical identification of these cells. The ventral aspect of the brain appears to be a major site for colonisation by microglia from E15 in the mouse. Cells within these basal areas appeared in a more advanced morphological state of differentiation, with several processes. These were interspersed between the developing cerebral vasculature. Thus, the differentiation of microglia coincided with the regional maturation of blood vessels, and progressed in a caudal-rostral gradient overall.

Of the immunohistochemical markers examined, only F4/80 was detected on populations of foetal murine microglia (**Figure 74**). F4/80 antigen is a 160Kd cell surface glycoprotein expressed by certain mononuclear phagocyte populations (McKnight et al. 1996). Molecular cloning analysis has shown that F4/80 shows some dgree of homology to two protein superfamilies- the extracellular NH_2 terminal contains several EGF-like repeats, while the final third of the molecule shows homology to members of the 7-transmembrane hormone receptor family. Although a definite function has yet to be ascribed to this determinant, it is known to be downregulated by IFN-γ and in certain types of infection. Furthermore, it is not expressed on macrophages within T cell areas of lymph nodes and spleen. Immunohistochemistry with the antibody raised against F4/80 demonstrated microglial progenitors and amoeboid cells located in the meninges, within the ventricular zone and at junctions with the choroid plexus (**Figure 74A-E**). Expression of this marker was significant on amoeboid cells within the corpus callosum, internal and external capsules (bordering the caudate-putamen), and occasionally seen on paired cells which may have recently divided (**Figure 74G-K**). The small vessel-associated progenitor cells that were detected with tomato lectin however, could not be detected with F4/80 antigen. Early ramifying cells were immunoreactive for F4/80 in the caudate-putamen, preoptic area, dorsomedial and ventromedial thalamic nuclei, habenula, and within the VZ and SVZ. Bipolar cells were also detected within the marginal zone underlying the meningeal layer. However, expression of this marker was downregulated on these ramifying cells from E18 to E20, and they became more difficult to identify (**Figure 74 L-O**). Few of these early ramified cells could be detected at E20 or in newborn animals. The results indicated that F4/80 antigen, initially present on both amoeboid and early ramified cells from E15, was gradually downregulated with advancing gestational age. These observations support the view introduced earlier, that the embryonic period of microglial differentiation occurs between E17 and E19.

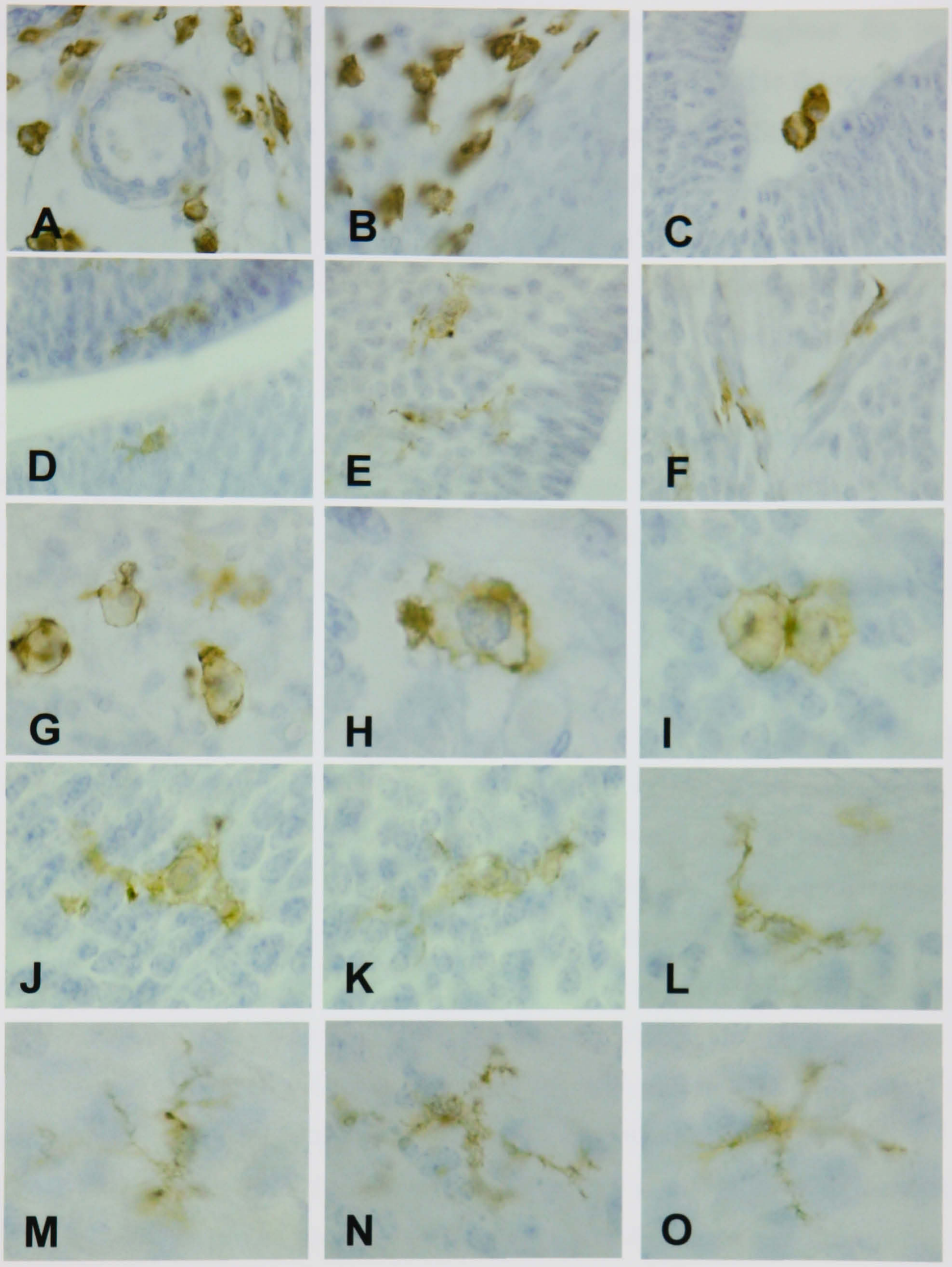


FIGURE 74

F4/80 immunoreactive microglia in the mouse brain, E15-E18

Immunoreactivity with F4/80 detected early progenitors surrounding meningeal vessels (**A**), at junctions between the choroid plexus and ventricular lining (**B**), occasional supraependymal cells (**C**), and within the ventricular zone (**D,E**). Very rarely, F4/80 immunoreactivity was also detected on cells attached to blood vessels (**F**). The small progenitor cells identified with tomato lectin however, could not be detected clearly. Instead, F4/80 was significantly expressed on amoeboid cells within the corpus callosum, internal and external capsules (**G-I**), where these cells were occasionally seen in pairs, having probably recently divided (**I**). On the whole F4/80 immunoreactivity was relatively weak on amoeboid microglia from E15 onwards. These early cells were in the process of differentiating (**J-K**). By E18 different morphologies were present from bipolar to multipolar forms (**L-O**). These were progressively more difficult to visualise as they were only faintly immunoreactive for F4/80 antigen. Fewer cells could be detected using F4/80 by comparison with lectin histochemical identification of microglia.

Expression of adhesion molecules and chemokines

Of the adhesion molecules examined, none were detected throughout the period of development under study. The finding that ICAM-2 was not expressed in the murine CNS was surprising, but important, since this may suggest species-specific differences in the recruitment of microglia to the CNS, or specialisation during cerebrovascular development. Curiously, the expression of MIP-1α was also not found within the developing mouse brain, which further questions its role in regulating human foetal microglia. However, MCP-1 expression was indeed noted within the embryonic mouse brain (**Figure 75**). MCP-1 expression was particularly evident at E19, and concentrated within the cortical plate (superficial aspect), meningeal layer, and the ventricular layer. Interestingly, similar to that found in the human foetal CNS, expression of this chemokine was confined to the lateral telencephalon and not detected in the frontal or occipital poles. Unlike the human foetal brain, MCP-1 was more intensely expressed in *superficial* cortical layers, rather than within the lower cortical plate and subplate.

Finally, the embryonic mouse brains were screened for the expression of granulocyte-monocyte colony stimulating factor (GM-CSF). MCSF and GMCSF are two growth factors considered essential for directing the lineage of myeloid cells to macrophages and granulocytes. MCSF is present in embryonic CNS and its receptor found on microglia (Chang et al. 1994). Recently, it has been found that microglia are still present, albeit reduced in numbers, in the brains of mice deficient in MCSF (Blevis and Fedoroff, 1995; Wegiel et al. 1998; Wiktor-Jedrzejczak and Gordon, 1996). Therefore, GMCSF, which is produced locally by astrocytes in the CNS, may in this respect be hypothesised as equally important for the differentiation of microglia. Immunohistochemical expression of GMCSF was not found within the CNS parenchyma during the embryonic period, and only weak immunoreactivity was detected within the meninges at the earlier time points examined (not shown). Therefore, further studies are necessary to evaluate expression of this growth factor within the developing CNS in relation to microglia.

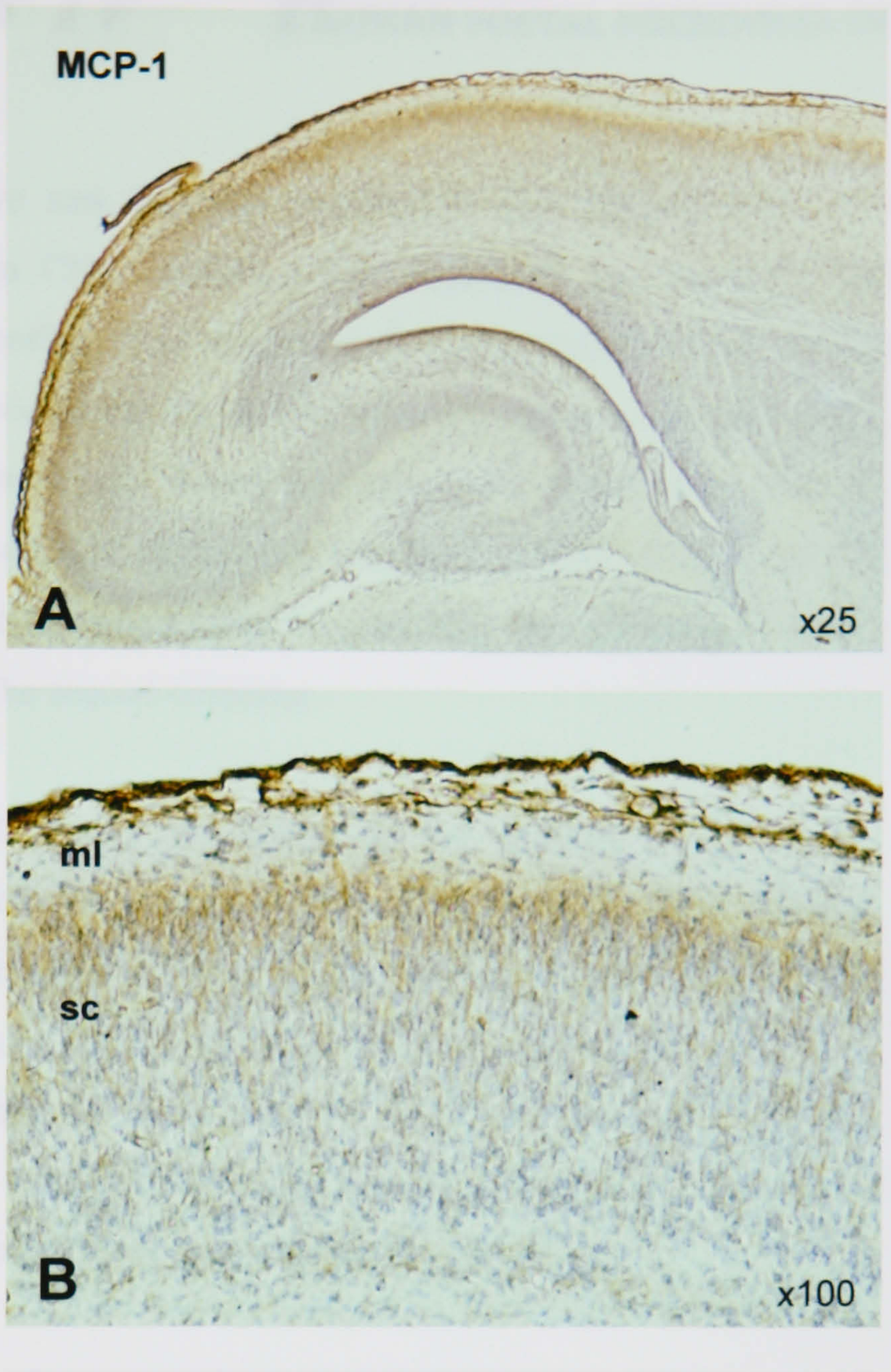


FIGURE 75

MCP-1 expression in the mouse brain at E19

Expression of MCP-1 was most noticeable in the cortical plate (particularly the superficial cortical plate, sc), within the meninges, and additionally on the ventricular lining/ ventricular zone. Furthermore, immunoreactivity within the cortical plate was mainly confined to the lateral neocortex. (**A**) Horizontal section of the brain at E19, showing the hippocampus and overlying cortex. Higher magnification of neocortex (**B**), to show immunoreactivity within the cortical plate.

Chapter IV HUMAN FOETAL MICROGLIA IN TISSUE CULTURE

In Chapter II, we saw that the distribution and differentiation of microglia within the developing human CNS coincided both regionally and spatially, with that for astrocytes during the same period. This suggested that a functional interaction may take place between these two cell types during the second trimester that may directly influence their proliferation and differentiation. It was shown that selective expression of chemokines (notably MCP-1, RANTES, MIP-1α) and chemokine receptors (CCR2, CCR5, CXCR4) within the human foetal CNS could be responsible for directing the colonisation and spatial distribution of microglia during the second trimester.

This Chapter will further investigate (i) the dynamic morphological interaction between human foetal microglia and astrocytes maintained in tissue cultures, (ii) characterise the phenotypical expression of specific chemokines (notably MCP-1, RANTES, MIP-1α) and chemokine receptors (CCR2, CCR5, CXCR4) by these two cell types in co-culture, and (iii) assess the response of these cells to select recombinant chemokines. The fundamental questions of how ramified microglia develop and maintain their regular spatial distribution were also examined in these tissue culture preparations.

The following questions are addressed:

- Is there a direct morphological transition between amoeboid and ramified microglia?
- How are microglial morphology and motility influenced by astrocytes in subconfluent compared to confluent cultures?
- Do human foetal microglia maintain their electrophysiological characteristics by demonstrating inward-rectifying K+ channels *in vitro*, and how does this compare with rodent preparations?
- Is the expression of certain chemokines in these co-cultures related to cellular interactions and the morphological transformation of microglia?

Materials & Methods

Human material

Primary human brain cultures, and bone marrow preparations were established from tissues obtained from elective pregnancy terminations at King's Healthcare NHS Trust. These were collected with approval of the ethical committees of the Bethlem, Maudsley and King's Healthcare NHS Trusts. Intact and dissected foetal (13-19 weeks of gestation) brain tissues. were collected, placed in holding medium (HM: HBSS (modified). 2mM glutamine. 10mM HEPES buffer, 20μg/ml gentamycin sulphate and transported to the laboratory for culture preparation within 2-3 hours after clinical collection. Gestational age (in weeks) was estimated according to the following criteria in order of preference: anatomically by crown-to-rump length, foot length, hand measurements and calculated from last menstrual period.

Primary human foetal CNS cultures

Primary human foetal microglia and astrocyte co-cultures and isolated cultures were established as previously described (Rezaie et al. 2002). We and others (Williams et al., 1992) have found that adherence properties of foetal and adult human microglial cells contrast those of neonatal rodent preparations where microglial cells are less adherent than astrocytes and thus can be floated off by rotary shaking. The rotary shaking method of McCarthy and de Vellis (1980), although cited on numerous occasions as suitable for rodent material, was found to yield too few microglial cells in our human foetal preparations. Human foetal brain macrophages/microglia were found to emerge from explant cultures grown on agar for 3-4 days without a change in medium and proliferated extensively (as described for rodent organ 'explant' cultures) see **Figure 76**. The modified technique of holding intact tissue in medium for 3 days prior to dissociation was therefore used as a pre-requisite for obtaining higher yields of microglia in this investigation. The basal culture medium employed RPMI 1640 supplemented with 100U/ml penicillin. 100μg/ml streptomycin, 2mM glutamine and 5% FCS.

Samples were not pooled between gestational ages. Instead. material from each sample was cultured in isolation to maintain homogeneity of cultures. Briefly, tissues were carefully dissected on a sterilised gauze, meninges and contaminating blood vessels micro-dissected and discarded. The entire intact tissue was then transferred to a $25cm^3$ sterile plastic tube containing culture medium and maintained in an incubator for three days. Cells were obtained in the absence of proteolytic enzyme digestion, as this allowed for immunophenotypic and functional characterisation without concomitant alteration in cell surface features associated with tissue processing (Ford et al., 1996; Havenith et al., 1998).

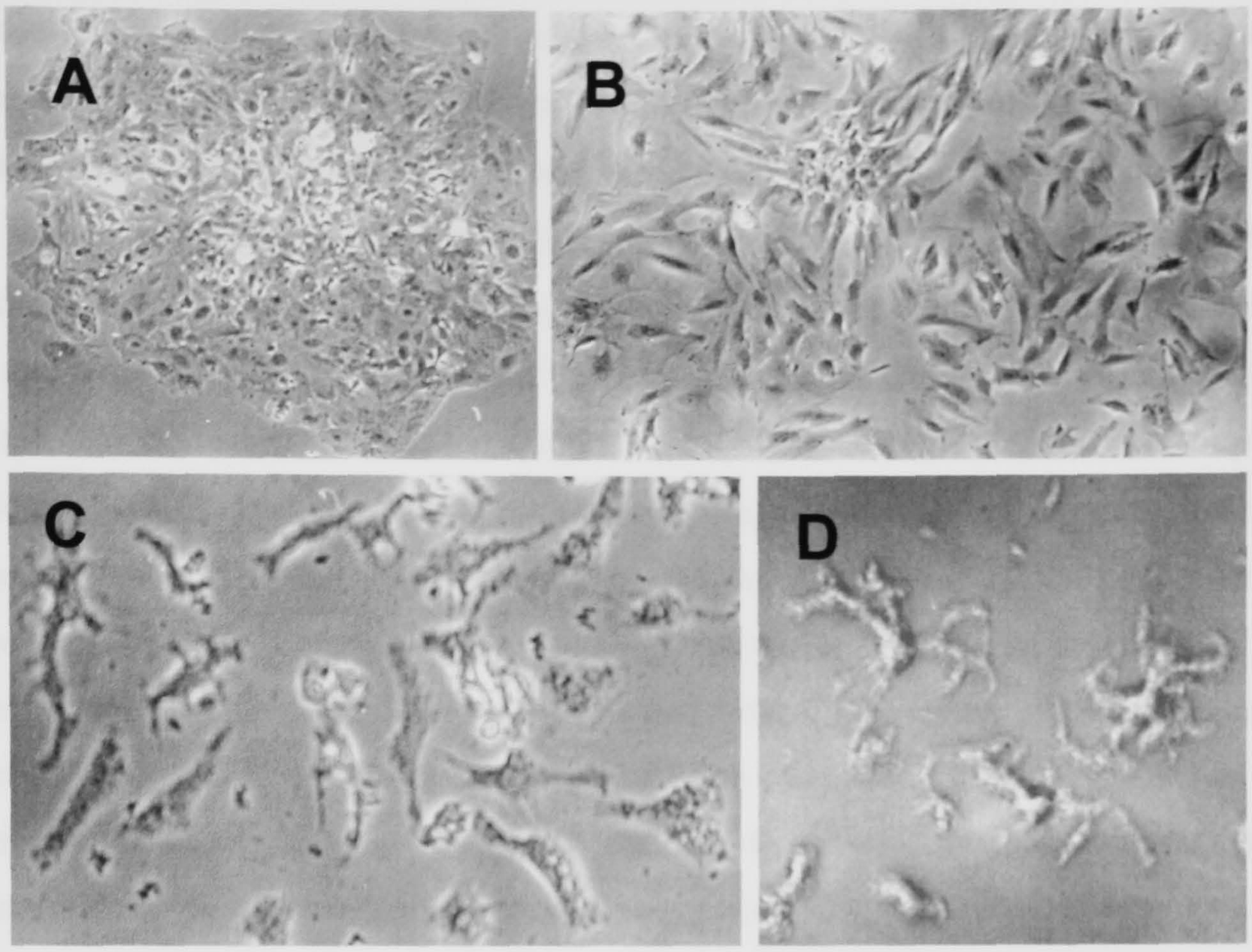


FIGURE 76

Human foetal CNS explant culture preparations cultivating microglia

Phase-contrast microscopic images of mixed glial cultures (**A,B**), enriched for activated (**C**) and progressively ramified (**D**) microglial cells.

As it was previously found that foetal CNS cultures were sensitive to digestive enzymes, repeated mechanical dissociation of cerebral cortical cells was carried out instead, using progressively finer fire-polished Pasteur pipettes to obtain isolated cell suspensions. 2 x 10^5 cells/ml were seeded in 25 or 75cm^3 flasks or on circular glass coverslips immersed in 12/24 well plates. Poly-l-lysine, laminin, fibronectin (10µg/ml) and primaera-coated flasks (Falcon/Nunclon) and coverslips were used for comparison of culture adherent cells. Cultures were maintained in RPMI 1640 medium (Sigma) supplemented with 5% heat-inactivated FCS (Sigma), 1% normal human serum (Sigma), 2mM glutamine, 1% penicillin/streptomycin solution (100U/ml penicillin, 100µg/ml streptomycin) and 2 x 10^{-5} M β-mercaptoethanol, in an incubator at 37°C. All solutions were filter-sterilised through a 0.2mm pore filter (which excludes the smallest bacterial contaminants ~0.8mm) prior to adding to the medium. The use of heat-inactivated foetal calf serum was of particular significance, since heat inactivation destroys complement, in order to prevent activation of mononuclear phagocytes in culture. Essentially, heat-inactivated serum is both antibody-free and complement-free. Likewise, all instruments and appliances were autoclaved and rinsed in endotoxin-free water to eliminate contamination and activation of cell cultures. The medium was replaced 50% every 3-4 days, and cultures maintained for up to six months.

Seeding density is known to influence cellular proliferation of microglia (Alliot et al. 1991). In all preparations a seeding density of 2 x 10^5 cells/ml gave consistent and reproducible numbers of microglia. The gestational age of foetal tissue also determined the relative ratio of microglia to astrocytes in our hands (i.e higher number of ameboid microglial cells derived from 14 weeks or earlier specimens). The above method was found to result in a pure co-culture of astrocytes (~95%) and microglia (~5%) over a period of four weeks, without contaminating oligodendrocytes or neurons, similar to that previously described in rodent CNS preparations (Giulian and Baker, 1986) and human preparations (2% microglia: Lauro et al. 1995). More purified (>99%) astrocyte cultures were established with prolonged incubation in culture and less frequent change of medium, and by passaging and removal of microglial cells by the rotary shaking method (these were harvested for isolated microglial culture preparations).

Characterisation of glial cultures

In order to define the cell populations isolated, cultures were fixed *in situ* with methanol and characterised immunocytochemically (**Table 6**) with antibodies to macrophages/microglia (CD11b, CD45, CD64, CD68), astrocytes (GFAP), and endothelial cells (PECAM-1, 1:00 dilution, Serotec, UK), and glial progenitors (O4, A2B5, 1:50-1:100 dilution, Biogenesis, UK) according to standardised protocols (Dako ABC-HRP method and Vector Alkaline-

phosphatase protocol). These produced brown-black (DAB), violet (Vector VIP) and red (Vector red) reaction products. Where appropriate the DAB chromogen eaction product was enhanced using Nickel or Cobalt chloride to give a dense blue-black precipitate. Additional immunoreactivity to PCNA (Serotec, UK) and was also assessed. Cytospin preparations were also used for cellular identification and characterisation, following dissociation of cells with trypsin-EDTA (Sigma, UK). Some samples were frozen *in situ* in FCS containing 10% DMSO, and subsequently stored at –70°C. These were used for electrophysiological verification (cell recovery 10-20%). Co-cultures were screened for expression of chemokines and chemokine receptors **Table 7**.

Immunocytochemistry

Many variations of immunocytochemical protocols are available for demonstrating reactivity *in situ* on frozen or paraffin-embedded sections. Our previous studies *in vivo* employed a modification of the DAKO-ABC-HRP method, described in detail in Chapter 2 (Rezaie et al. 1997; Simpson et al. 2000), which has now been adapted for cells in culture. Permeabilisation of cells (with a detergent such as Tween 20 or Triton X-100) is necessary in order to demonstrate detection of intracytoplasmic in addition to surface antigens. The protocol renders cells viable for immunoreactivity while preserving microstructure as viewed using conventional light microscopy. For negative controls the primary antibody was omitted from the procedure and cells were incubated with normal sera alone. Frozen tissue from the CNS of two cases diagnosed with multiple sclerosis served as positive controls (Simpson et al. 2000). Digitised pictures of cell cultures were captured as *.tif format files and processed using a video camera mounted onto a Zeiss Axioplan microscope and OPTIMAS v6.0 image analysis software for windows.

Time lapse video microscopy

In viable cultures, microglia were identified under phase-contrast microscopy on the basis of morphological characteristics (determined from previous immunostained preparations). Culture flasks were placed in a temperature (37°C), humidity and CO_2 regulated chamber mounted onto the stage of a microscope (Zeiss Axiovert 135M) with a gas lamina flow attachment. Images were recorded using a CCD videocamera connected to a black and white monitor via a time-lapse video cassette recorder (Panasonic AG-6720, recording speed range from 0 to 480 hours). Live microglia were identified in culture fields under low magnification according to characteristic morphologies. Individual cells or a group of cells in close proximity were selected for study at higher magnification. The video recorder was set to 1/160 speed (i.e. a 3 hour tape converted to 480hours) for recording of cultures. Frames were simultaneously captured using a conventional camera. Individual sequences of images were

subsequently converted to *.tiff files from video tapes using a Windows-based editing software (Mirovideo Studio 200 for Windows 95 (video image frame grabber), Video Director 3.0, Pinnacle Systems Inc. USA). These were used for analysing various parameters. For confirmation and to identify the cell types, the same cells that were analysed by video time-lapse were immediately fixed by replacing medium with 100% methanol, and processed for immunocytochemistry to monocyte/macrophage markers CD11b/CD45/CD64/CD68 (microglia) and GFAP immunoreactivity (astrocytes), as previously outlined.

Analysis of process length, area occupied and motility of microglia

Measurements of the extent of ramification (total length and number of processes), 2-dimensional areas occupied by cells and motility of microglia in the co-cultures were investigated using the OPTIMAS programme, version 6.0 (OPTIMAS corporation, USA). The software was initially calibrated to the dimensions of the screen image (μm) for subsequent frame by frame sequence analysis of area, extent of ramification and velocity. Area morphometry was calculated from individual contour images of the cells using a digital recording pen. The total length of processes was determined by summation of the lengths obtained from a constant central point on the cell to the most distal tips of processes.

The 'analysis of motility/determination of velocity' utility on the OPTIMAS programme enabled tracking of objects through a time-sequenced series of images and computed direction, distance travelled, position and velocity for each object tracked. The velocity of microglia in co-cultures was determined using a correlation-based analysis of contour images individually and precisely outlined with a digital recording pen. Contour images were computed and compared at defined time intervals. The optimum fit of successive image sequences resulted in the localisation of the cell (given as x and y co-ordinates) from which the migrated distances (μm) and velocities (μm per hour) were calculated. The overall velocity of a given cell was also presented as the mean value for all time points during the experiment. Motility was defined as retraction/protraction of processes, or through comparison of individual cell bodies at the beginning and end of each experiment, when a cell had migrated a distance of at least more than one cell body diameter in any direction. All analyses were performed in the absence of growth or additional factors (medium alone).

Treatment of glial cultures with recombinant human chemokines and bacterial LPS

Effect of recombinant human MCP-1 and MIP-1α on astrocyte proliferation

Tissue samples from human foetal CNS at 19 gestational weeks were cultured for 8-9 weeks in 75cm^3 plastic flasks until astrocytes attained full confluence. Microglia were depleted using the rotary shaking method and cells were trypsinised and passaged onto round glass coverslips

coated with poly-l-lysine and inserted in 12 well plates. These were allowed to adhere for 72 hours, prior to treatment with 0.5. 2.5, 10 and 50ng/ml of recombinant human chemokines (diluted in HBSS containing 0.1%BSA) for 18 hours [rhMIP-1α (10μg/ml lyophilised) Sigma Chemical Co., Ltd; rhMCP-1(10μg/ml) R&D systems, Europe]. Cells were fixed with methanol and stained with Haematoxylin (light microscopy) or Hoechst 33342 (fluorescence microscopy), and nuclei were counted in 20 random non-overlapping fields at x200 magnification, using a 10x10 grid eyepiece graticule. All assays were carried out in duplicate, and data given as the mean number of nuclei per 200x field ± SEM.

Effect of recombinant human chemokines and LPS on foetal CNS cultures

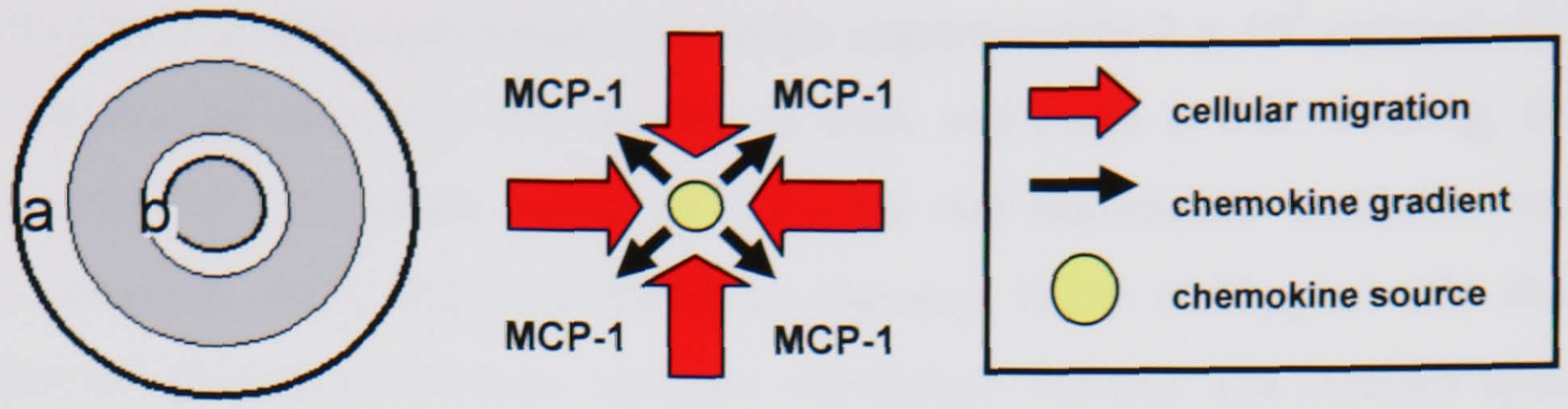
Samples of CNS material from 13, 14 and 15 gestational weeks were processed for tissue culture as described. Glial cells were passaged onto coverslips in 12-well plates for seven days and treated with 0.5, 2.5, 10 and 50ng/ml recombinant human chemokines or 5, 50, 500 and 5,000ng/well LPS for 18 hours. Cells were fixed in methanol and stained immunocytochemically for MCP-1 and CCR2. Immunoreactivity from triplicate assays was graded, and carried out in direct comparison with baseline expression of MCP-1 and CCR2 in unstimulated cultures and in control tissue sections.

Assessment of the response of human foetal microglia and astrocytes to recombinant human chemokines using the Dunn chemotaxis chamber

Attempts were also made to assess directed migration of human foetal glial cells in response to recombinant human MCP-1 and MIP-1α using the Dunn chemotaxis chamber (Weber Scientific International, Teddington, Middlesex) (Allen et al. 1998; Webb et al. 1996; Zicha et al. 1991, 1997). This system allows the migration, persistence and directionality of cells to be monitored in relation to the direction of the chemoattractive gradient, in more stable and linear concentration gradient of chemoattractants than can be achieved using Boyden chambers. The Dunn chemotaxis chamber is essentially a modified Helber counting chamber slide. Cells are initially cultured onto coverslips that are then inverted onto the slide. Those cells that rest on the annular bridge of the chamber (the coverslip is separated by a thin gap of no more than ~20μm from the slide, due to the continuity of fluid between the outer and inner wells), can be observed using phase-contrast and fluorescence microscopy, and their migratory tracks can be recorded using automated time-lapse video analysis or frame-by-frame sequence analysis under fluorescence optics. The normal procedure to set up gradients, would require both concentric wells of the chemotactic chamber to be filled with chemoattractant-free medium, and the coverslip seeded with cells inverted onto the chamber in an offset position, allowing access to a narrow slit at one edge for refilling the outer well. After firmly seating the coverslip, it would be sealed into place using melted dental wax. The medium would next be

drained from the outer well and replaced with medium with or without chemoattractant. Cells placed in a concentration gradient (for example macrophages responding to MCSF), will polarise and migrate towards the source of chemoattractant.

The following modifications of this procedure were used in this study: 1. 10 and 100ng/ml of recombinant human chemokines: MIP-1α (Sigma, UK) and MCP-1 (R&D systems, UK) were made up in RPMI 1640, and 20μl of each placed in the inner well of separate chemotaxis chambers (**Figure 77**). In the initial attempts 80μl of a fluorescence-labelled cell suspension was placed in the outer wells and a round 16mm coverslip overlaid onto the chamber. These cells had been dissociated from confluent co-cultures or isolated preparations of human foetal microglia and astrocytes, by first washing in HBSS (lacking in calcium and magnesium), detached from the flask using trypsin/EDTA, and the cell suspensions spun and washed with HBSS (non-modified). They were next labelled in suspension with a weak RCA-1 solution (1:10,000 RCA-1 conjugated to rhodamine (excitation wavelength 570nm, emission 595nm) for 30 minutes, Vector Laboratories, UK) or 1:500 dilution of PG-M1 conjugated to FITC (for 30 minutes, Dako UK, excitation wavelength 490nm, emission 520nm), spun down and washed twice with HBSS, before being labelled with the cell viability marker calcein AM (1:500 dilution for 30 minutes, Molecular Probes). Calcein AM (excitation wavelength 495nm, emission 520nm) is among the most reliable indicators of cell viability from a number of recognised dyes that label live cells in tissue culture (including carboxy fluoresceindiacetate/CFDA, fluoresceindiacetate/FDA, BCECF/AM, acridine orange). It is reported to display superior retention by mammalian cells and its green-yellow fluorescence is insensitive to pH. Calcein (the hydrolytic product of calcein AM) is a polyanionic fluorescein derivative with six negative and two positive charges at pH7. Specifically, unlike the other dyes, calcein AM does not interfere with leukocyte chemotaxis, superoxide production, or affect lymphocyte conjugation with target cells (Callewaert et al. 1991; DeClerck et al. 1994; Denholm and Stankus, 1995; Mandeville et al. 1995; Racliff et al. 1991). The leaching of calcein from loaded cells has been used as a measure of membrane permeability in response to temperature (Bischoff et al. 1995), cellular damage or assays of cytotoxic T lymphocyte activity which disrupts the integrity of cellular membranes (Lichtenfels et al. 1994).



Dunn Chemotaxis Chamber

FIGURE 77

Schematic illustration showing the principle behind the application of the Dunn Chemotaxis Chamber

Cells deposited in a well located around the periphery (**a**), migrate along a flat, raised platform (shaded grey) along a chemotactic gradient towards the central source of a chemoattractant (**b**). Alternatively, isolated microglial cells cultivated on a glass coverslip (coated with laminin, fibronectin, poly-L-lysine) or microglia co-cultured with astrocytes can be overlaid onto the chamber and the rate of migration towards or away from the central source can be estimated from a series of images captured over a set time-frame.

Next cells were spun down and washed in HBSS, reconstituted in pure RPMI 1640 medium and counted using a haemocytometer to obtain approximately 2 x 10^5 cells/ml. In order to further substantiate determine the viability of cells, and assist in their tracking. cell nuclei were stained with propidium iodide (0.125μg/ml, red fluorescent, excitation wavelength 530nm, emission, 615), Hoecshst 33342 or Hoechst 33258 (0.25μg/ml UV fluorescent, excitation wavelength 355-365nm. emission wavelength 465nm). The Hoechst dyes bind to the minor groove of DNA at A-T rich sequences. Hoechst 33342 permeates cells more rapidly than Hoechst 33258, and is commonly used for determining the DNA content of viable cells without detergent treatment or fixation (Arndt-Jovin and Jovin, 1977; Shapiro, 1989). Data obtained with Hoechst 33342 correlates less well with the red fluorescent propidium iodide which is a cell-impermeant nucleic acid (DNA/RNA) stain (Myc et al. 1992). Hoechst 33342 is initially taken up by apoptotic cells, whereas cell-impermeant dyes such as propidium iodide and ethidium bromide are excluded. Later stages of apoptosis are accompanied by an increase in membrane permeability, which allows propidium iodide to enter cells. Therefore, a combination of Hoechst 33342 and propidium iodide can be used for analysing the stages of apoptosis and cell cycle distribution (Belloc et al. 1994; Curnow et al. 1994; Ormerod et al. 1993; Sun et al. 1992)

In subsequent experiments, the same labelling procedures were used on cells cultured onto poly-L-lysine or laminin-coated glass coverslips, and these were overlaid directly onto the chemotaxis chamber. All assessments were made at room temperature, in the dark, and in triplicate. Photographs of fluorescent cells over defined areas of the platform that formed a bridge between the inner and outer wells (shaded grey in **Figure 77**) were captured manually at 15-20 minute intervals over a period of up to 6 hours. At the end of experiments, cells were fixed with 1% paraformaldehyde solution for 30 minutes, and coverslips mounted onto a slide using fluorescence mounting medium (0.42g glycine, 0.021g sodium hydroxide, 0.51g sodium chloride, 0.03g sodium azide, dissolved and made up to 30ml in water, to which was added 70ml glycerol).

Electrophysiological recording of glial cells

Human foetal mnicroglia and astrocytes (prepared from samples at 13 and 17GW) which had been previously stored frozen at -70°C were rapidly thawed at 37°C and transferred to a universal tube, diluted dropwise in approximately 10ml of RPMI 1640 medium. They were spun and washed twice in HBSS, prior to transferring to a culture flask in the presence of supplemented RPMI 1640 culture medium (as described, with the exception that 10% heat-inactivated foetal calf serum was used for re-culturing these cells). They were cultured for 72 hours and seeded onto glass coverslips for electrophysiological recording. Glial cultures were

also prepared from the cerebral cortex of newborn Wistar rats. Cortical tissues were carefully freed of meninges and blood vessels, trypsinised for two minutes, and dissociated with a fire-polished pipette, and washed twice. Mixed glial cells were cultured for 9-12 days in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal calf serum and antibiotics. Medium was changed every three days. Microglia were separated from underlying astrocyte monolayers by gentle rotary shaking (100 rpm) of the flasks for 1 hour in an incubator at 37°C. Cells collected in the supernatants were seeded at a density of 4 x 10^4 cells/well onto glass coverslips.

Electrophysiological recordings were conducted under the guidance of Drs. Christiane Nolte, Carsten Ohlemeyer and Clemens Boucsein in the laboratory of Professor Dr. Helmut Kettenmann, Berlin, January 1997. Recordings were made using the conventional patch-clamp technique in the whole-cell configuration (Boucsein et al. 2000). Coverslips with cells were placed in a chamber which was continuously perfused with bath solution ([134mM] NaCl, [2.5mM] KCl, [1.3mM] $MgCl_2$, [2mM] $CaCl_2$, [1.25mM] K_2HPO_4, [26mM] $NaHCO_3$; [10mM] D-Glucose) at a flow rate of 4-6 ml/minute. The electrode solution contained [130mM] KCl, [2mM] $MgCl_2$, [0.5mM] $CaCl_2$, [2mM] Na-ATP, [5mM] EGTA, [10mM] HEPES. Recordings were made using microelectrodes pulled from borosilicate capillaries with filament (6-8MΩ). Cells were monitored using live video camera output to a black and white monitor, as the pipette was advanced under microscopic view. Pressure was applied to the pipette once it was resting on a cell, as indicated by an increment in oscilloscope readout. Non-compensated currents were recorded with conventional electronics (EPC9 amplifier, HEKA electronics, Germany) at a sampling rate of 3 KHz, and signals filtered at 2.9KHz. Microglia were clamped close to their membrane potential of -70mV (ranging from -40mV to -70mV holding potential, for all glial cell types recorded), for recording membrane currents. Membrane currents could be reliably recorded for up to one hour when cells were clamped at a holding potential of -40mV for astrocyte/oligodendrocytes and their precursors and -70mV for microglia. Membrane currents were activated by stepping of voltage for 50-75 msec to depolarising and hyperpolarizing potentials from the holding potential. Nine steps, each with an increment of 10mV were performed in each direction (i.e. for microglia, down to -160mV: depolarising, up to +20mV: hyperpolarising potentials). Recordings are presented in Figure 82.

Results

Morphology, electrophysiological properties and phenotype of human foetal microglia maintained in culture

Microglia in isolated preparations were typically found to adopt unipolar, bipolar, amoeboid, and less frequently, multipolar morphologies over a period of 1-2 weeks following initial cultivation (**Figure 78**). However, the 'amoeboid' variety in isolated cultures tended to be more noticeably flattened than rounded in appearance. These cells rarely survived beyond the second week without the addition of growth factor supplements to the medium. Isolated human foetal microglia adhered to laminin, fibronectin, poly-l-lysine and primaera-coated flasks with similar adhesive properties. There was no significant variability in morphology associated with substratum. However, microglia in these cultures extended long threadlike processes of small diameter. More stable 'thorny' projections were emitted at regular intervals from pseudopodial extensions or from the cell body area. These were reminiscent of 'thorny projections' on microglia reported *in vivo*. All varieties were capable of phagocytosis of cellular debris as evidenced by engulfment of particles and accumulating granular vesicles within the cytoplasm. However, astrocyte-conditioned medium derived from the supernatant of confluent astrocyte cultures failed to promote ramification of isolated microglia in these cultures.

Human foetal microglia and astrocytes derived from material between 13 and 19 weeks gave consistent homogeneous cultures. With progressive time in culture, astrocytes formed confluent monolayers with motile microglia occupying distinctive areas above or beneath the astrocytes. In contrast to the uniformity of confluent astrocytes, microglia were heterogeneous in shape (amoeboid, bipolar, ramified). When co-cultured with subconfluent astrocytes, microglia tended to preferentially interact and associate with these cells and adopted an amoeboid morphology in the initial stages of cell culture (**Figure 78** and **Figure 79**). This association occurred between individual cells and there was occasional evidence of cellular division as a result of this union (**Figure 79F**). Nevertheless, microglial cell numbers did not vary considerably in these non-stimulated cultures, with only very occasional cells expressing PCNA, as a marker of proliferation (S-phase of cell cycle). Attachment to astrocytes involved several stages (**Figure 79G-L**), whereby amoeboid microglia migrated along astrocytic processes towards the cell body area where they adopted a perinuclear position overlying the solitary astrocyte and progressively ramified in this location, as the culture reached confluence.

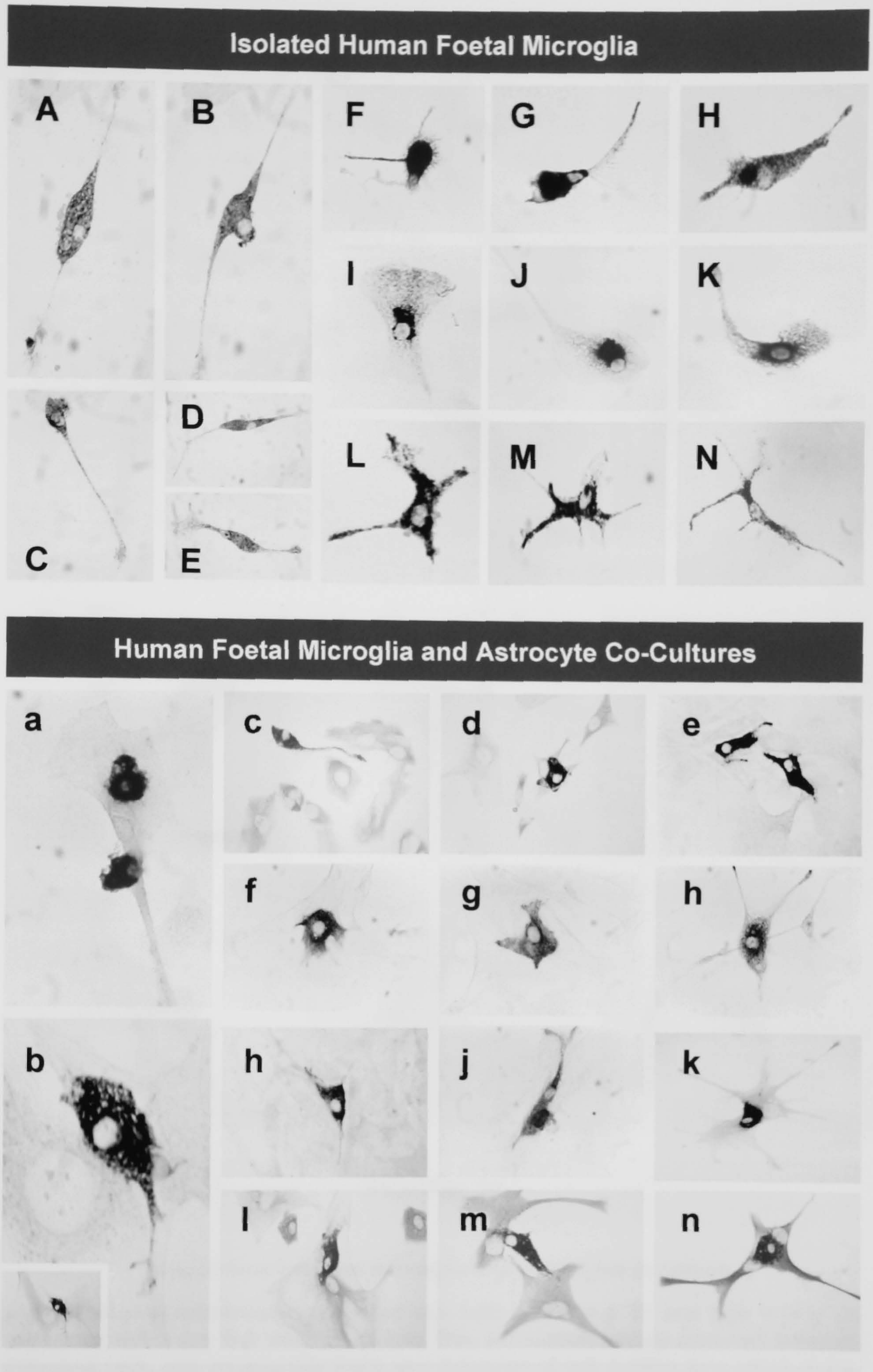


FIGURE 78

Morphology of human foetal microglia maintained in isolated culture preparations (top panel) and in co-culture with foetal astrocytes (lower panel)

Light microscopic images of CD45:CD68 immunostained microglia in isolated preparations (**A-N**), and in non-confluent astrocyte co-cultures (**a-n**).

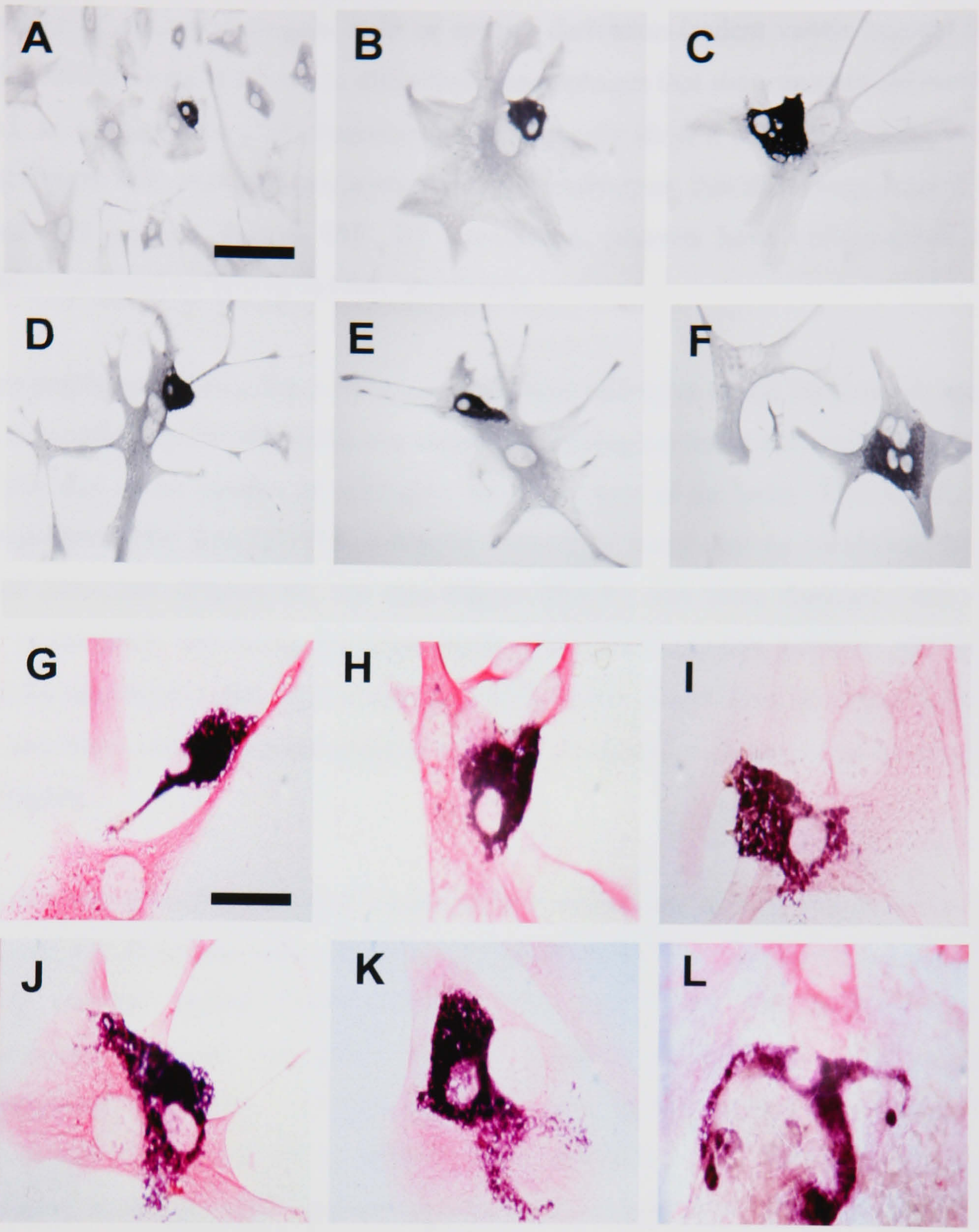


FIGURE 79

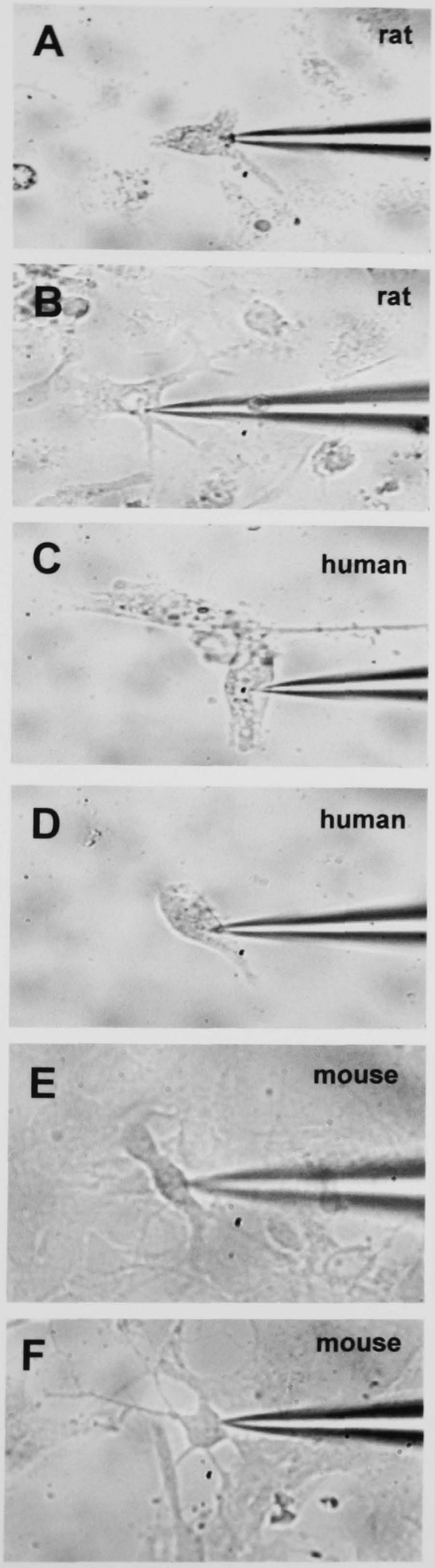
Interactions between microglia and astrocytes in culture

(**A-F**) Microglia preferentially associated with both type I (e.g. B) and type II (e.g. D) astrocytes within the first week in culture This association usually occurred between individual cells, and occasionally there was indication of cell division as a result of this union (**F**). Microglia interacted with astrocytes in a stereotypical manner as shown in **G-L**, and underwent progressive ramification with confluency of astrocytes in co-culture (**K-L**). Note the perinuclear position adopted by microglia overlying astrocytes. Scale bar represents approximately 200μm in A, 40μm in B-F, 20μm in G-L.

Microglia maintained in tissue culture possessed an inward rectifying potassium current, irrespective of the morphological state or species derivation (rodent versus human) (**Figure 80A-D**). This property is known to differ from macrophages that show an outward current (not shown), as well as from glial precursors which typically show a delayed outward rectifying current **Figure 80E**, and from astrocytes and oligodendrocytes. that show large passive inward and outward currents **Figure 80F**. By comparison, neurons have voltage-gated sodium channels, not found on microglia (data not shown)

In more confluent cultures, the morphologies of foetal microglia varied from round/ amoeboid forms to bipolar, tripolar and multipolar varieties overlying confluent astrocytic layers (**Figure 81**). Estimates of the number of microglial sub-types were of the order: 75% amoeboid, 12-15% bipolar/tripolar and 12-15% multipolar. Microglia could also be identified below the layer of astrocytes (**Figure 81**, see also **Figure 89A-F**), and these flattened cells roamed freely, occasionally extending sheetlike motile projections upwards between astrocytes, as viewed by time-lapse video microscopy. Microglia varied in size from 30-50μm in diameter. Some amoeboid cells displayed granular vacuolar cytoplasm with numerous lysosomes and lipid droplets.

Astrocytes and microglia displayed considerable heterogeneity in their phenotype, but could be consistently identified using antibodies to GFAP and CD68 respectively. A monoclonal antibody cocktail against CD11b, CD45, CD64 and CD68 was used to detect all morphological subtypes (amoeboid, bipolar, ramified) of microglia. The progressive ramification of these cells took place over a period of 1-2 months in co-cultures with astrocytes and depended on the gestational age of sample material. O4 and A2B5 positivity was minimal or absent in these cultures, as was immunoreactivity to PECAM-1, as an intrinsic endothelial marker during development. GFAP immunoreactivity varied between astrocytes. Generally, large flattened (type I) cells displayed weak/moderate staining whereas the less frequent type II cells and glial precursors in earlier cultures (smaller rounded cells with delicate processes) were intensely positive. Purified astrocyte cultures occasionally displayed parallel arrays of astrocytic processes that resembled radial glia found *in situ* (not shown) These eventually however. gave rise to confluent monolayers of flattened type I astrocytes (lacking processes) within a period of two weeks.



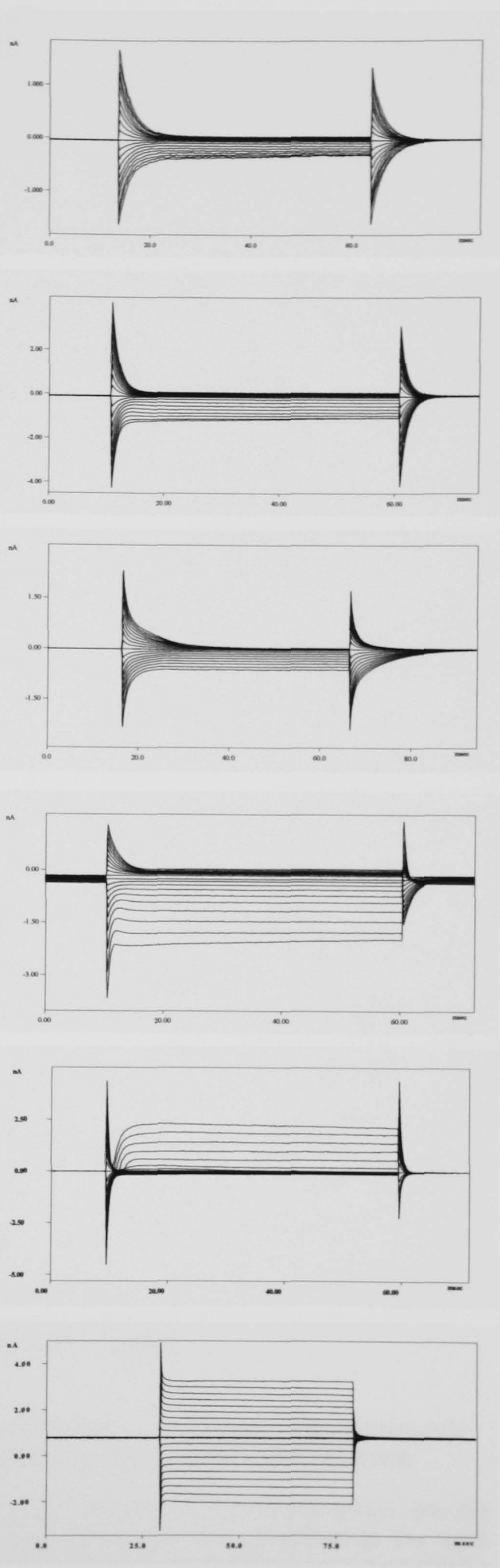


FIGURE 80

Electrophysiological recordings characteristic of microglia, oligodendrocyte precursors and oligodendrocyte/astrocytes in culture preparations

FIGURE 80 *(continued)*

Electrophysiological recordings characteristic of microglia, oligodendrocyte precursors and oligodendrocyte/astrocytes in culture preparations

The panels on the left show the morphology of cells undergoing the procedure, and the corresponding membrane currents (nA) versus time (ms) are presented to the right. Membrane currents were activated by stepping of voltage for 50-75 msec to depolarising and hyperpolarizing potentials from the holding potential. (**A-D**) Microglia maintained in tissue culture possessed an inward rectifying potassium current as indicated, irrespective of the morphological state or species derivation (rat versus human). (**A,B**) postnatal rat microglial preparations, (**C,D**) human foetal microglial preparations. A delayed outward rectifying current is a characteristic typical of oligodendrocyte precursor cells (E). Differentiated oligodendrocytes/astrocytes (F) display typical large passive inward and outward currents.

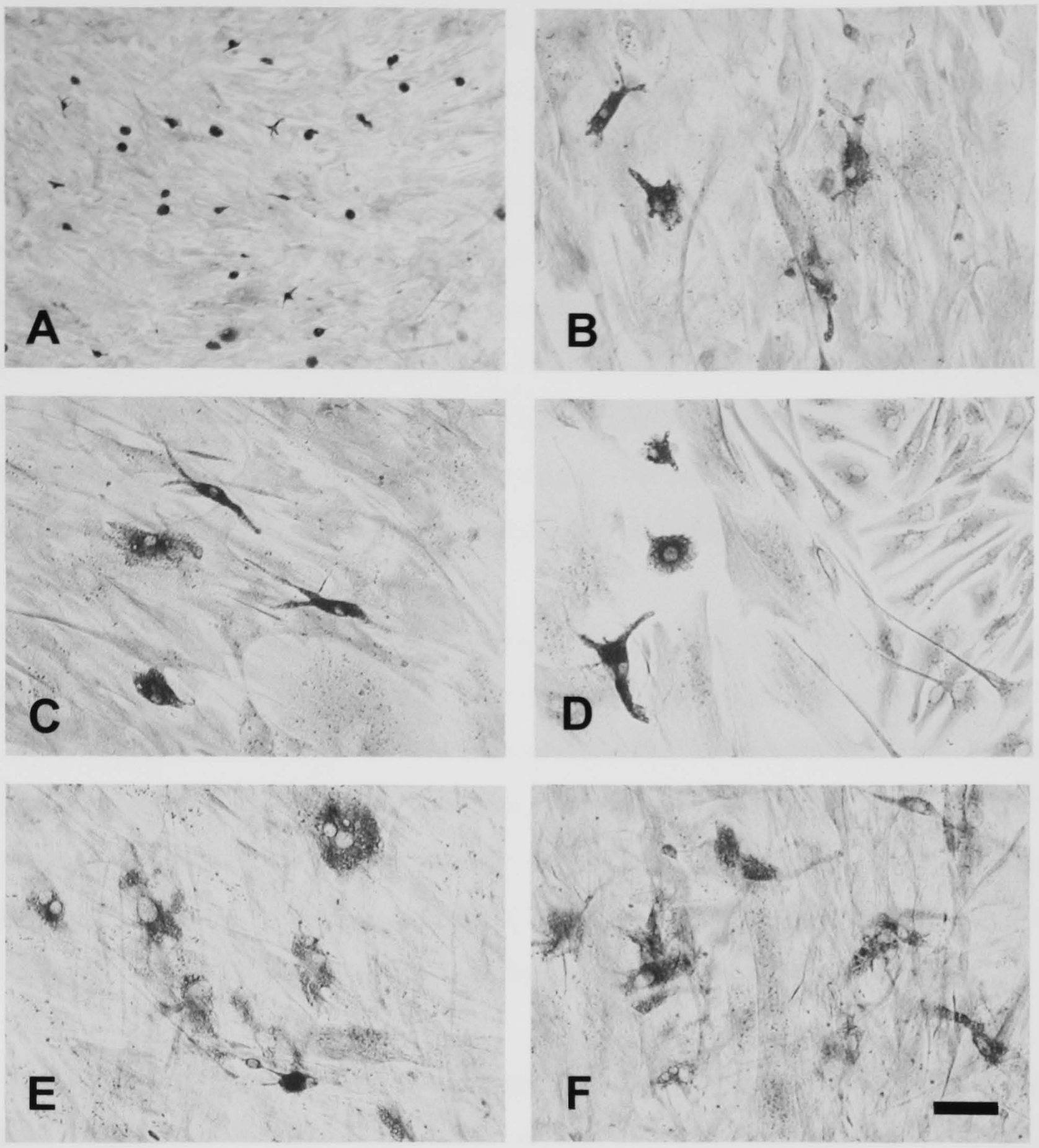


FIGURE 81

Human foetal microglia and astrocyte co-cultures

Microglia can be identified both above (**A-D**) and below (**E,F**) the confluent layer of astrocytes. CD45/68 positive cells overlying astrocytes tend to adopt amoeboid (approx. 75%), bipolar/tripolar (~12-15%) or multipolar (12-15%) forms, whereas those underlying the astrocytic layer appear flattened due to the confined space. These cells roam freely below the astrocytes and often extend sheet-like, motile projections upwards, between the astrocytes, which can be viewed clearly with time-lapse video footage. Occasionally, amoeboid (rounded) microglia can be seen within open spaces that occur between astrocytes (**D**). Cultures were established from samples obtained at 15 gestational weeks, cultured for 7-8 weeks. Scale bar represents approx. 250μm in A, and 20μm in B-F.

Motility and ramification of human foetal microglia

Microglia were in a continuous state of motion, and the morphological and motile states of these cells were dictated by the state of confluency of astrocytes in co-culture. The remarkably motile nature of microglia was best appreciated using time-lapse video microscopy. More than 50 microglia in a variety of morphological states were recorded, and seven are presented here (**Figures 82-88**). Pseudopods were continuously protracted and retracted as the cells moved incessantly within a defined area and underwent a variety of morphological transformations (**Figure 82**). Individual microglial cells in subconfluent cultures underwent a series of transitions from ramified to bipolar and ameboid forms associated with migration and differentiation of astrocytes in the immediate vicinity, all within a period of 24hours (**Figure 83**). For clarity, this has been presented in a schematic form in **Figure 84**.

Some cells demonstrated trailing processes very firmly adhered to astrocytes (**Figure 82A-F**). Consequently as microglia progressively moved away, trailing processes became elongated further until a point where they were retracted and other processes extended outwards to continuously monitor the culture environment (**Figure 82i-vii**). These could extend up to 100μm beyond the surface of the cell body and appeared to arise from retraction of the trailing edge of the cell at points of strong attachment to the substratum. Fragments of these thread-like processes could be frequently observed strewed across the substrate in culture.

Active phagocytosis by microglia was indicated by the accumulation of phase-bright and phase-dark vesicles (to a lesser extent even in ramified forms of microglia). Cytoplasmic vesicles diminished with progressive time in culture, as they were cleared by the cell. Vellum-like sheets of lamellipodia demonstrated continual exploratory movement both above and below the astrocytic layer, clearing cells of debris. Pseudopods were extended at passing particles of debris that were subsequently engulfed and internalised. Even in a distinctly ramified form, microglia navigated via the cell body and continuous protraction and retraction of processes (**Figure 86**, **Figure 87**). However, ramified microglia on confluent layers of astrocytes were more stable with respect to directional migration, and tended to occupy distinct 'territories' (**Figure 86**). Cell bodies of ramified cells varied between 10-20μm in diameter. The state of ramification appeared to be temperature-dependent: if the temperature of the culture was reduced to ambient room temperature (approximately 21°C), ramified microglia rapidly retracted their processes and adopted amoeboid morphology.

Occasionally astrocytes were seen to pull away from microglia. These cells displayed different motile characteristics from microglia, resembling fibroblasts or epithelial cells in their behaviour: they appeared as flattened fan-like cells and advanced with rippling/ruffling

membranes and evident cytoplasmic streaming. At the leading margin of the cell, lamellipodia were protracted with a smooth fluid motion, pausing occasionally for brief retrograde movements. Astrocyte cultures eventually established extensive sheets of flattened cells and moved only at their boundaries. In contrast to the rapid astrocytic proliferation, dividing microglia were not seen under time-lapse analysis.

Microglia in sub-confluent cultures displayed highly motile, amoeboid-intermediate ramified morphologies, progressively transforming to stabilised, quiescent ramified cells in co-culture with confluent astrocytes. Calculations of area, total length of processes and velocity are presented graphically (**Figure 85** and **Figure 87**) for the corresponding cells shown in **Figure 83** and **Figure 86**. Mean velocities (μm per hour) for individual cells are presented in **Figure 88**. The average mean velocity for cells in the intermediate-ramified form was calculated as 29.4μm per hour (range from 20.2 to 35.0μm per hour), equivalent to approximately 705μm per day. The cell with the greatest number of processes was also slowest with respect to directional motility (20.2 μm per hour). In contrast, the cell with greatest velocity (82.8 μm per hour) displayed the least number of processes. The rate of microglial cell motility also varied to some extent with temperature (personal observations), although this requires further investigation. Pearson correlation analysis revealed that the area of a cell correlated with the total length of its processes (e.g. cell 3: r^2=0.5, $p<0.01$; cell 5 r^2=0.24, p=0.016). There was an inverse correlation between the maximum length of processes and velocity of a cell (e.g. cell 4: r^2=-0.48, $p<0.001$; cell 6: r^2=-0.25, p=0.016). This confirms the hypothesis that cells lacking processes (in the amoeboid state) are capable of more rapid migratory responses compared to slower cells in the ramified state.

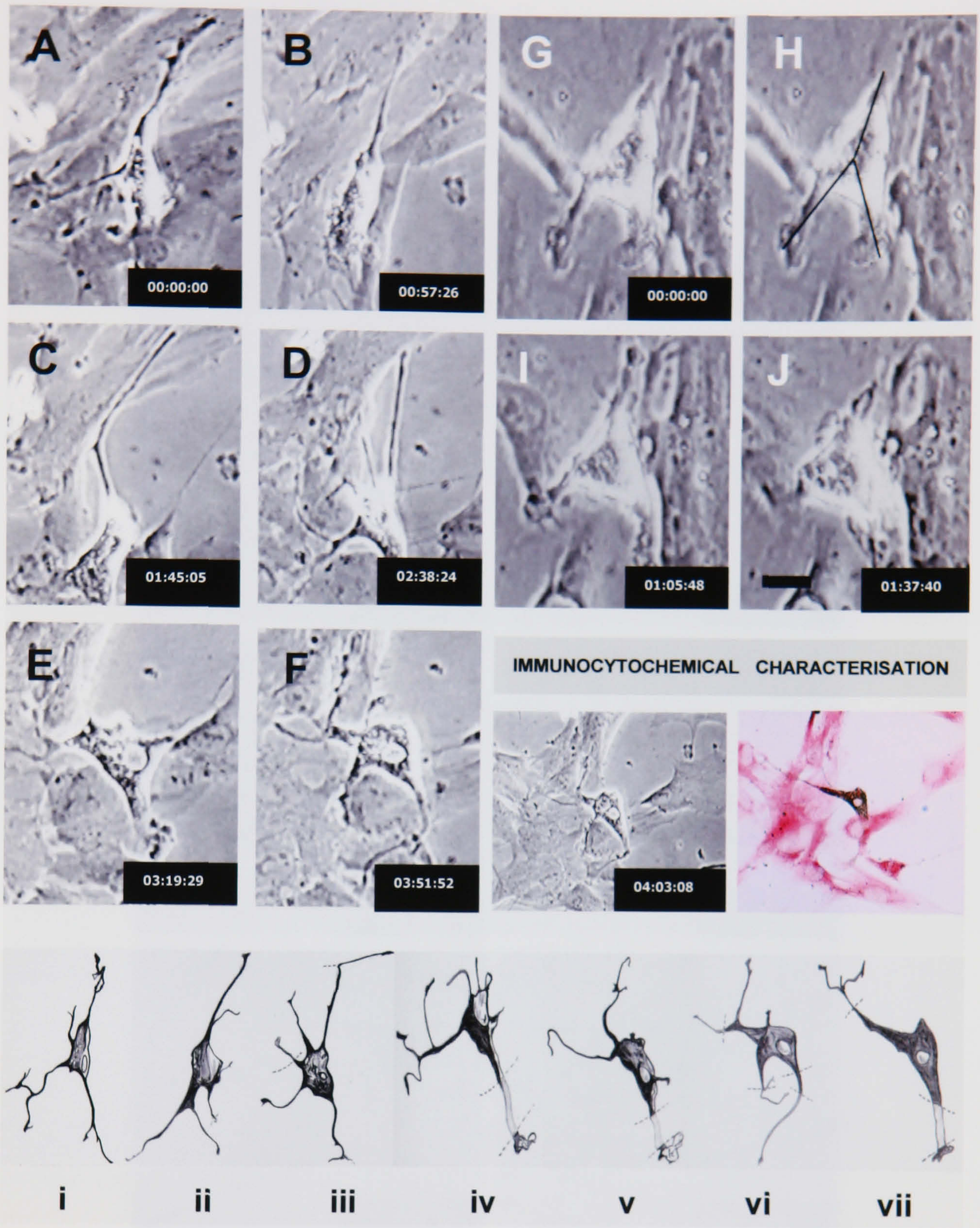


FIGURE 82

Morphological transformations of amoeboid/ramified microglia in co-culture with astrocytes (cells 1 and 2).

(**A-F**) Time-lapsed images of a ramified microglia and (**G-J**) an amoeboid/tripolar microglial cell overlying astrocytes in cultures established from CNS at 19 gestational weeks, maintained for 12 days *in vitro*. Elapsed time is given in hours: minutes: seconds. The cell shown in (**F**) was fixed *in situ* at 4 hours and 3 minutes following recording and processed for immunocytochemical identification (CD45:68 positive microglia, brown; GFAP-positive astrocytes, red). The morphological transformations of the microglial cell shown in A-F are presented pictorially (**i-vii**), over a period of 4 hours in culture. The intersection of processes with astrocytes (overlying or underlying) are shown as dotted lines. Note in particular the points of contact of the process emitted from the top end of the cell in (**iii**) and the vellum-like process that scouts below the astrocyte layer in (**iv-vii**). Bar represents approximately 25 μm in A-J.

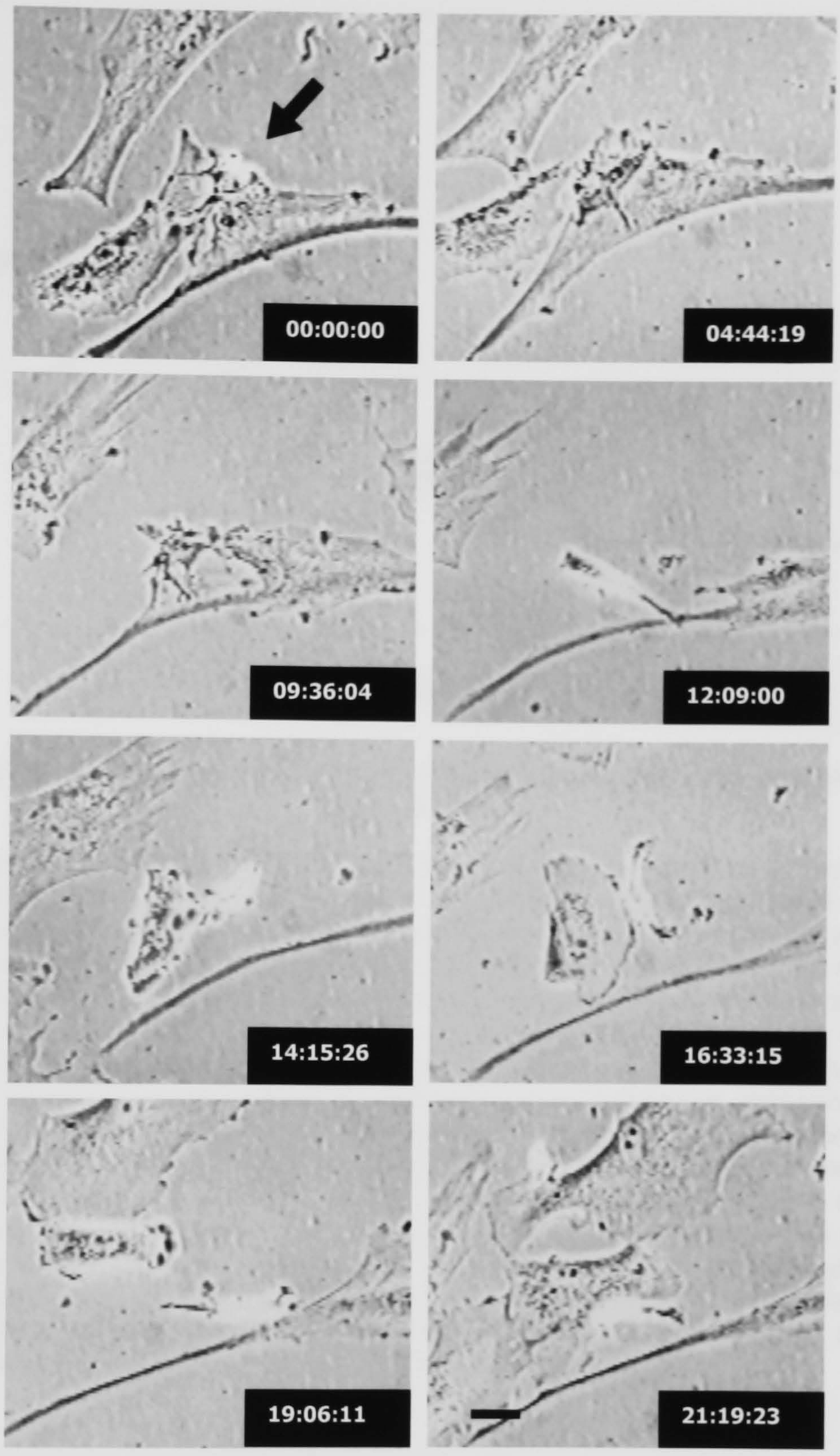


FIGURE 83

Morphological heterogeneity and motility of an individual microglial cell in co-culture with astrocytes, over a 24-hour period (cell 3).

(**A-H**) Time-lapsed images of a microglial cell (indicated by arrow in A) to demonstrate transformation from a ramified state overlying an astrocyte (**A**) to bipolar (**D**,**F**) and amoeboid (**H**) forms. Morphological transformation was clearly associated with the migration and differentiation of astrocytes in the immediate vicinity (**E-H**). Co-cultures were established from CNS obtained at 16 gestational weeks, maintained for 14 days *in vitro*. Elapsed time is given in hours: minutes: seconds. Scale bar represents approximately 30 μm.

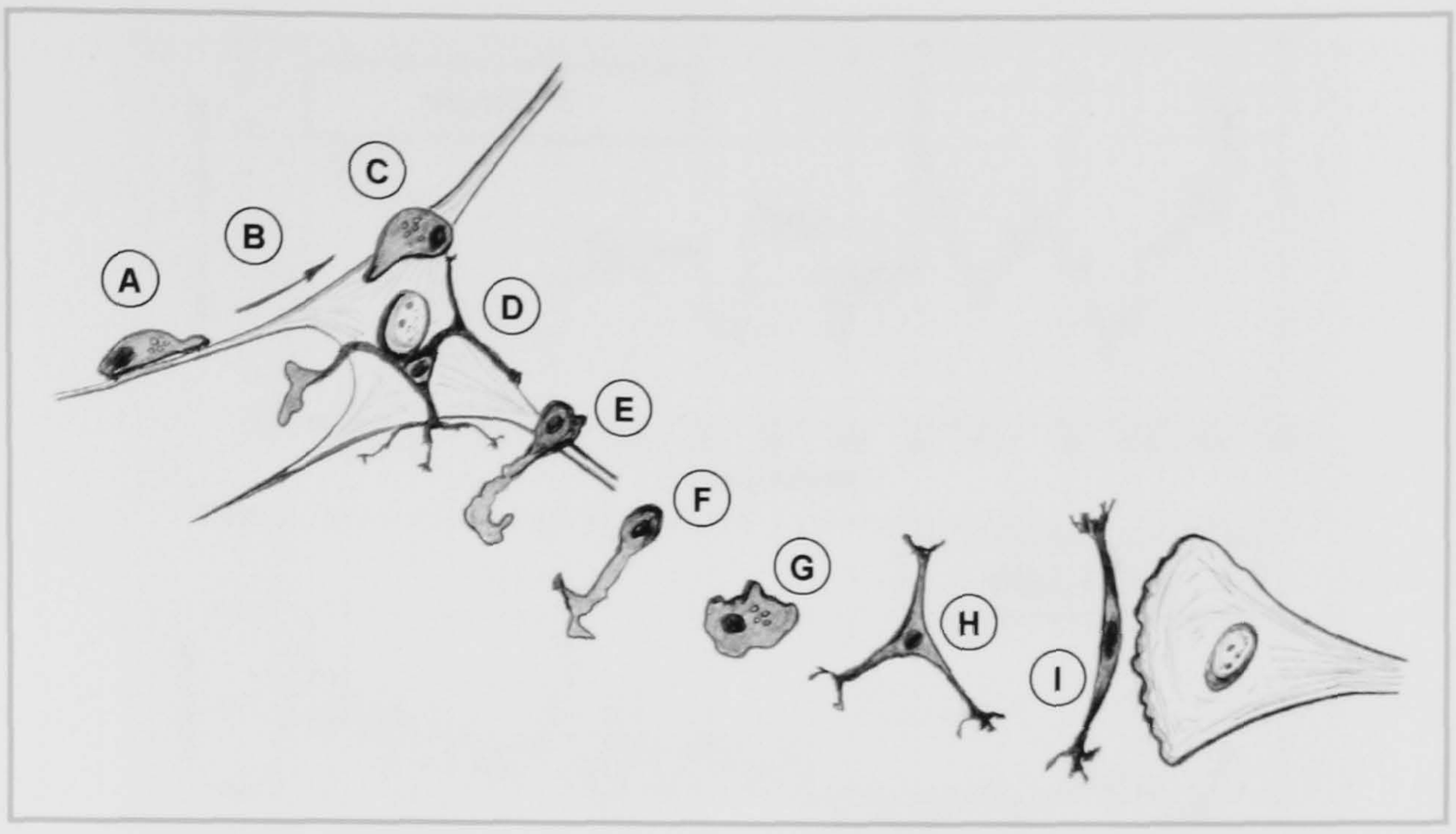


FIGURE 84

Stages in morphological transitions of human foetal microglia co-cultured with astrocytes

(**A-I**) Images taken form time-lapsed recordings of the morphological transformation of a microglial cell (shown in A), and typical of the interactions that take place between microglia and astrocytes maintained in sub-confluent co-cultures. In (**A**), an amoeboid microglia associates with a well-differentiated astrocyte, (**B**) migrates along an astrocytic process, (**C**) is positioned overlying the astrocyte cell body, (**D**) ramifies on top of the astrocyte, while its vellum-like and finer processes extend and monitor both the underlying astrocyte and the local environment. Eventually, (**E**) the microglia retracts its processes (de-ramification), with the exception of a vellum-like extension which continues to monitor the immediate environmental milieu via protracted circular motions and retraction. (**F**) The cell disassociates from the astrocyte, adopts (**G**) amoeboid, (**H**) tripolar, and (**I**) bipolar morphologies when isolated. An interaction between a migrating astrocyte and the bipolar microglial cell is shown to the right of the figure. The microglia extends its cell body region towards the ruffled membrane of an astrocyte which advances towards it. This astrocyte, subsequently undergoes rapid differentiation and settles down, adopting 'resting' characteristics typical of the original astrocyte encountered by the microglia (illustrated to the top left of the figure).

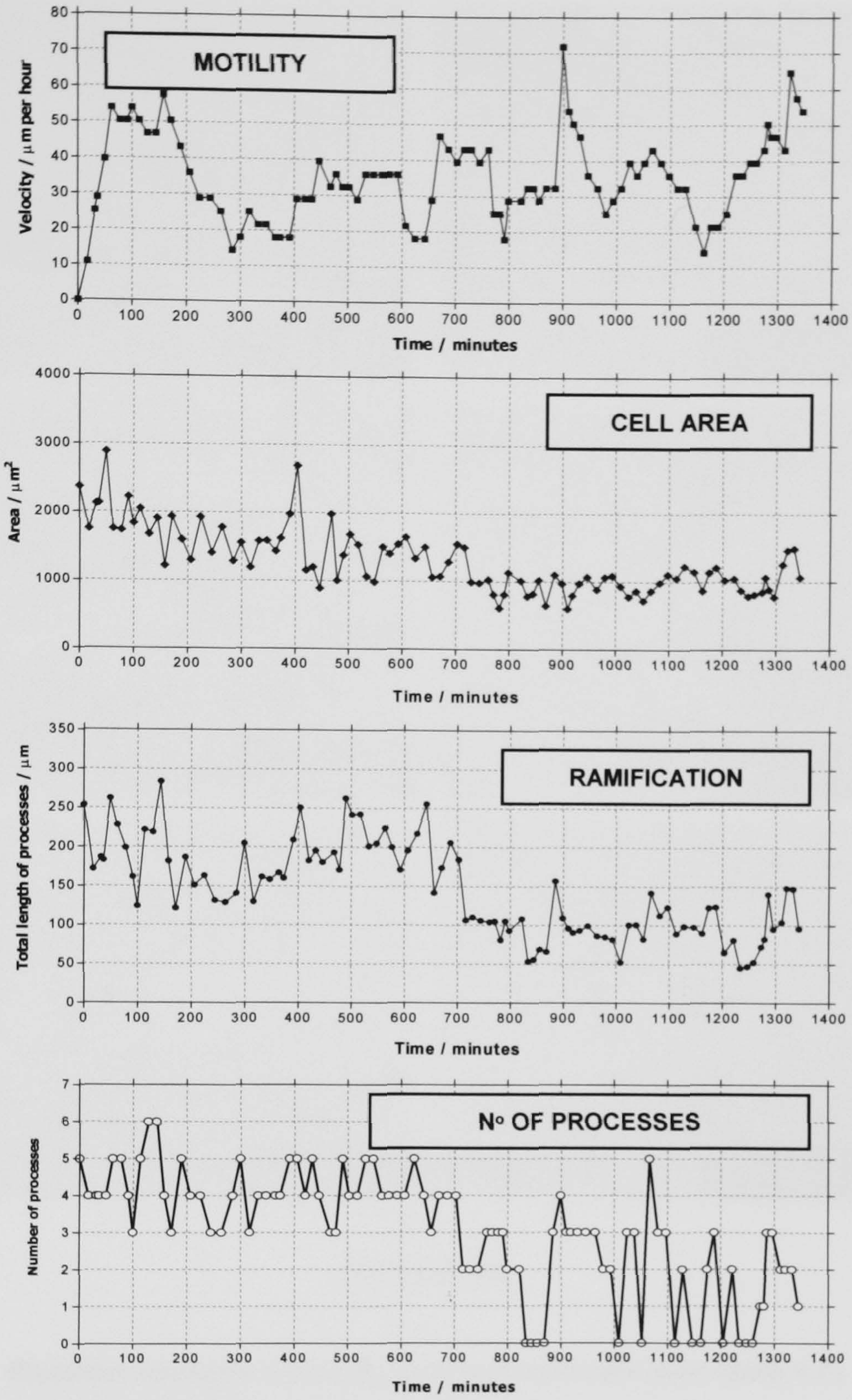


FIGURE 85

Morphological heterogeneity and motility of an individual microglial cell in co-culture with astrocytes, over a 24-hour period (cell 3).

The velocity, area occupied, ramification (total length of processes) and number of processes of cell 3 (shown in Figure 83), were analysed over a period of 24 hours. Note that the velocity and emission of processes correspond with the morphological state of the cell. The microglial cell overlying an astrocyte was initially ramified when measurements were recorded. Towards the end of recording however (800min+), the cell adopted characteristically tripolar, bipolar and amoeboid morphology, with rapid alterations in movement and protraction and retraction of cellular processes. Recordings for the total process length for the cell represent the summation of the maximum length to the distal tip of each process from a constant central reference point on the cell soma. Length recordings for the cell in amoeboid configuration represent the maximum diameter.

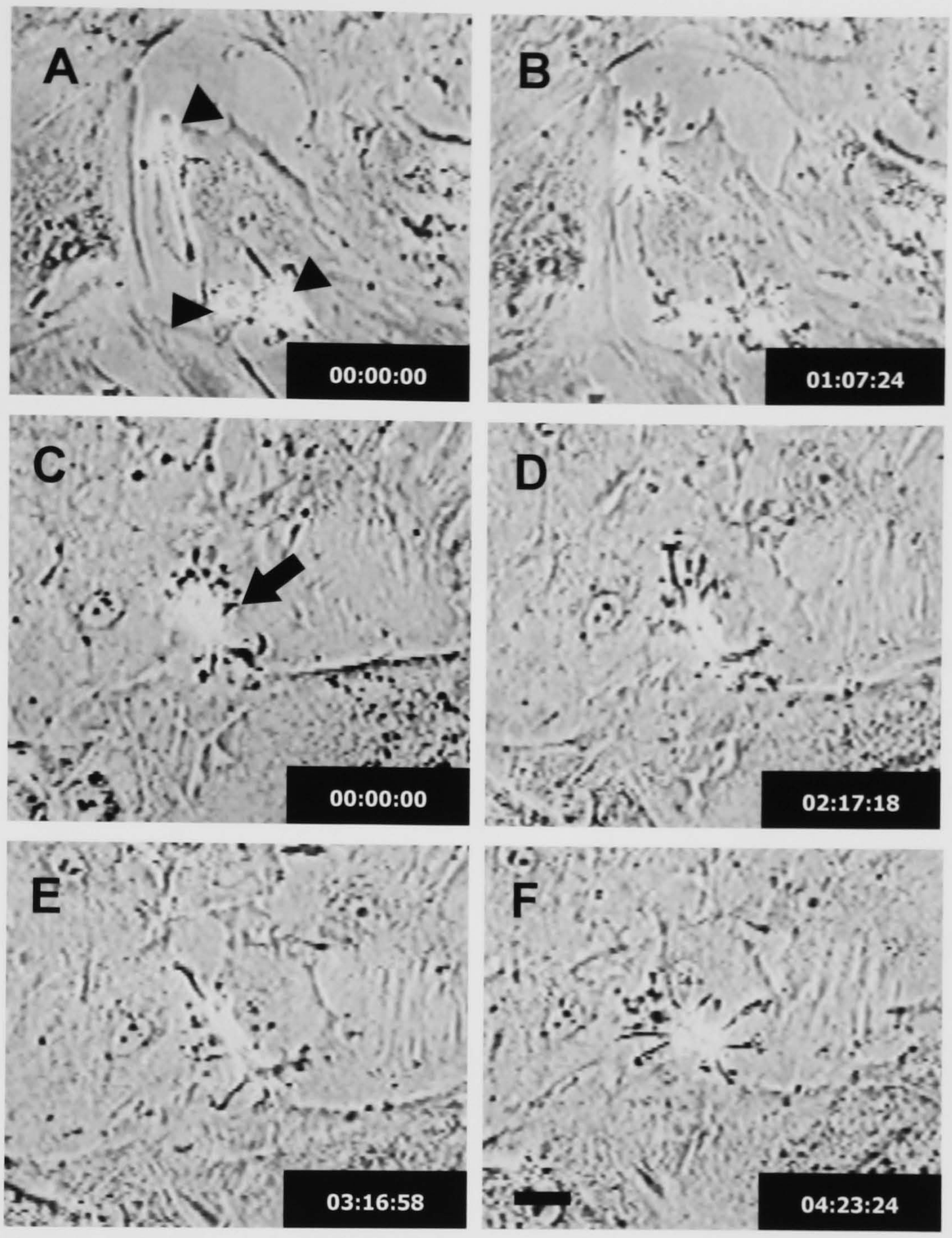


FIGURE 86

Ramified microglia overlying confluent astrocyte layer (cells 4-7).

Time-lapsed images of ramified microglia displaying numerous processes. Co-cultures were established from CNS obtained at 16 gestational weeks, maintained for 34 days *in vitro*. Three microglial cells (arrowheads) are shown in (**A**) and (**B**) and a single multipolar microglia (arrow) is shown in (**C-F**). These microglia occupied distinct 'territories' on astrocyte monolayers, with limited directional movement over a period of a few hours. However, continuous rapid protraction and retraction of processes was characteristic of these cells. In the ramified state, microglia adopted a defined territory within the center of the field. Despite the lack of directional movement, active motility of these cells could be observed as they continuously protracted and retracted their processes in various directions. Elapsed time given in hours:minutes:seconds. Bar represents approx. 25 μm.

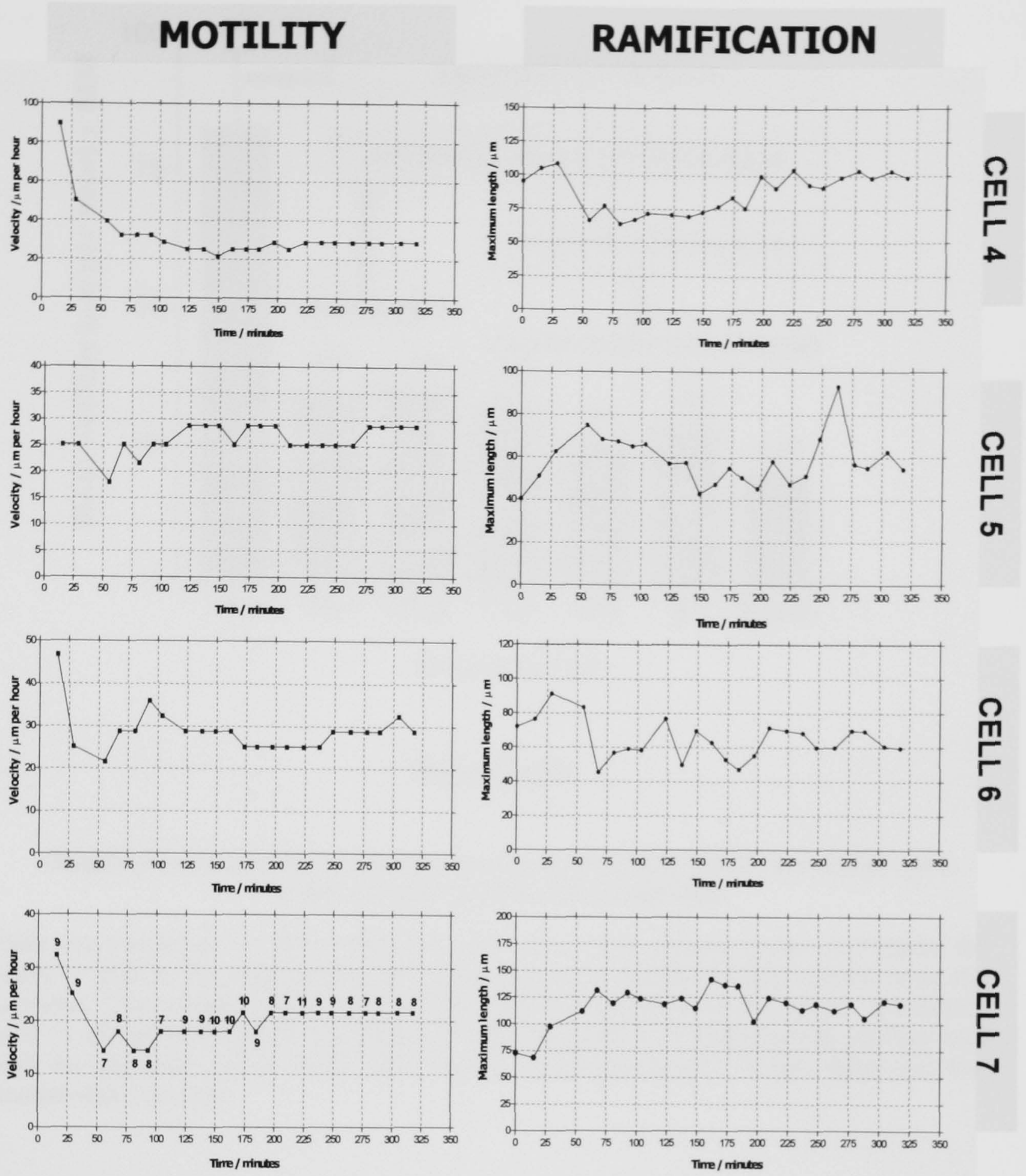


FIGURE 87

Graphical presentation of microglial velocity and extent of ramification compared between ramified cells on confluent astrocytes (cells 4-7).

The data correspond to cells shown in Figure 86. In their fully ramified state, human foetal microglia displayed a more stable baseline motility (left panel) in keeping with their morphology. However, there was continuous dynamic retraction and protraction of processes even in this 'resting' state (right panel, ramification). The corresponding number of processes for cell 7 are shown on the velocity graph (lower left panel).

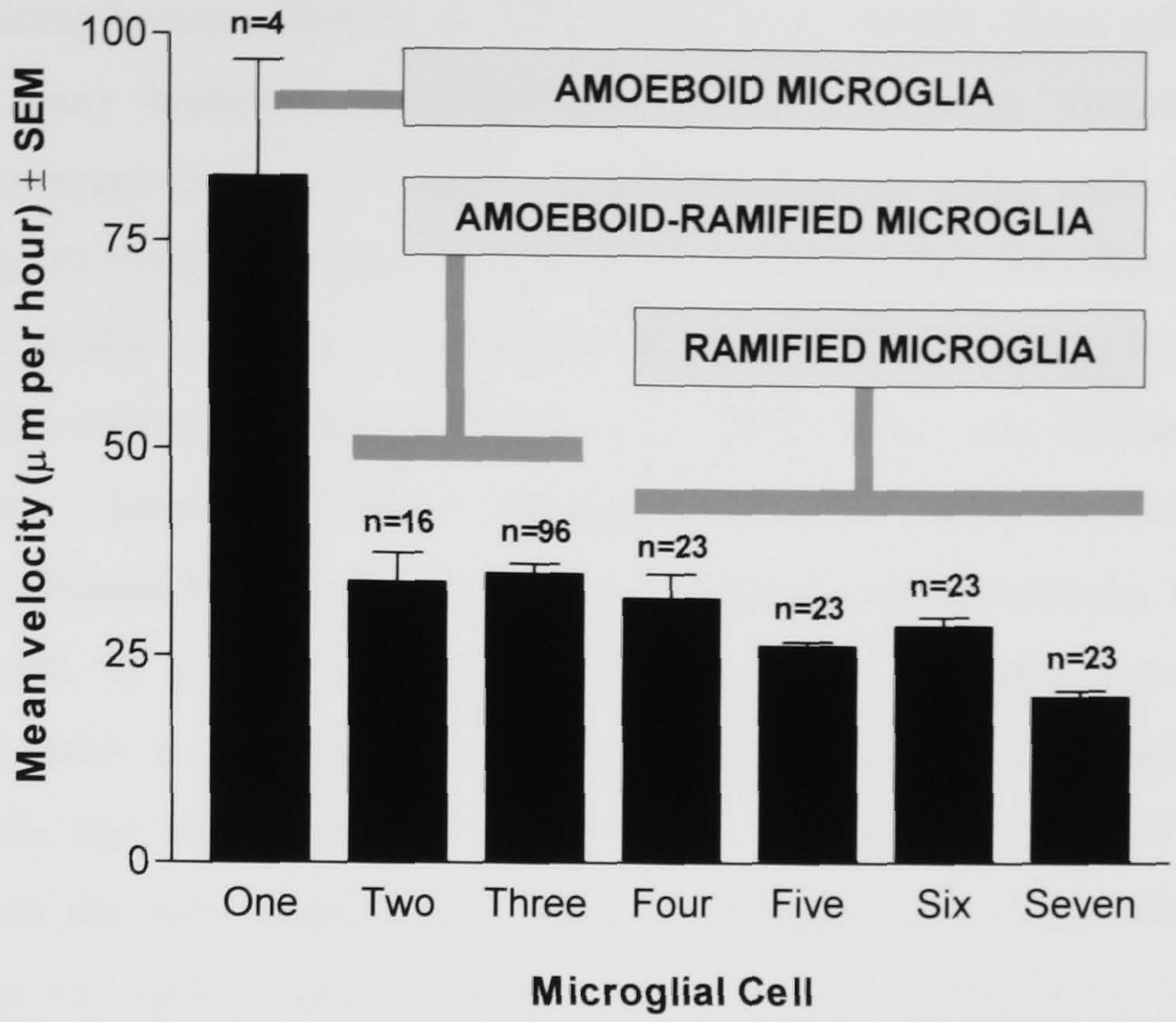


FIGURE 88

Graphical presentation of the mean velocity of human foetal microglial cells observed and recorded in co-cultures

Data are presented as mean velocity (μm/hour) ± SEM of cells shown in Figures 82 (cells 1 and 2), 83 (cell 3) and 86 (cells 4-7). *n* = number of time points used for analysis. The morphological states of the cells are indicated on the graph: amoeboid (cell 1), amoeboid-ramified (cells 2 and 3), ramified (cels 4-7). Note that the ramified cell with the greatest overall length and number of processes (cell 7) also displayed the lowest mean velocity.

Progression of cultures and detachment of microglia from confluent astrocytes

Ramified microglia were obvious in older cultures (>1 month). These cells possessed a small cell soma, many branched processes and lacked cytoplasmic vacuoles. These ramified microglia corresponded to a minor population of the total cells in culture (2-5%). Occasionally, ramified cells were situated within an open space between astrocytes in culture, but more frequently observed overlying the monolayer, and occupying distinct fields. This has been noted for rodent co-cultures (Glenn et al. 1989; Jordan and Thomas, 1987). In longer-term cultures, isolated and flattened microglia were additionally detected beneath astrocytic monolayers (**Figure 89A-F**). Those overlying the astrocytes eventually detached from these confluent cells by first retracting their processes, and adopting bipolar then amoeboid morphology prior to detachment (**Figure 89G-L**). The entire process took place within approximately one and a half hours as noted in time-lapse footage (**Figure 90**). A small proportion of the cells underwent a form of controlled cell death within 45-90 minutes, whereby the cell first became rounded, showed marked cytoplasmic blebbing, which was followed by nuclear rearrangement, fragmentation and rapid cell expansion. So far it has not been possible to detect whether this form of cell death is 'apoptosis', since TUNEL reactivity is negative in cultures. Prior experience from a number of pathological diseases, has shown that similar terminal morphological stages of macrophages also occur *in situ* (**Figure 91**). These cells can be found to accumulate cytoplasmic vesicles, and frequently at sites of greatest pathology, they can be found with a distended cell body, where intracellular components including the nucleus have been displaced to the periphery, or are altogether absent. These presumably defunct cellular 'remnants' probably await clearance within the tissues. Experimental validation of this phenomenon is currently under consideration.

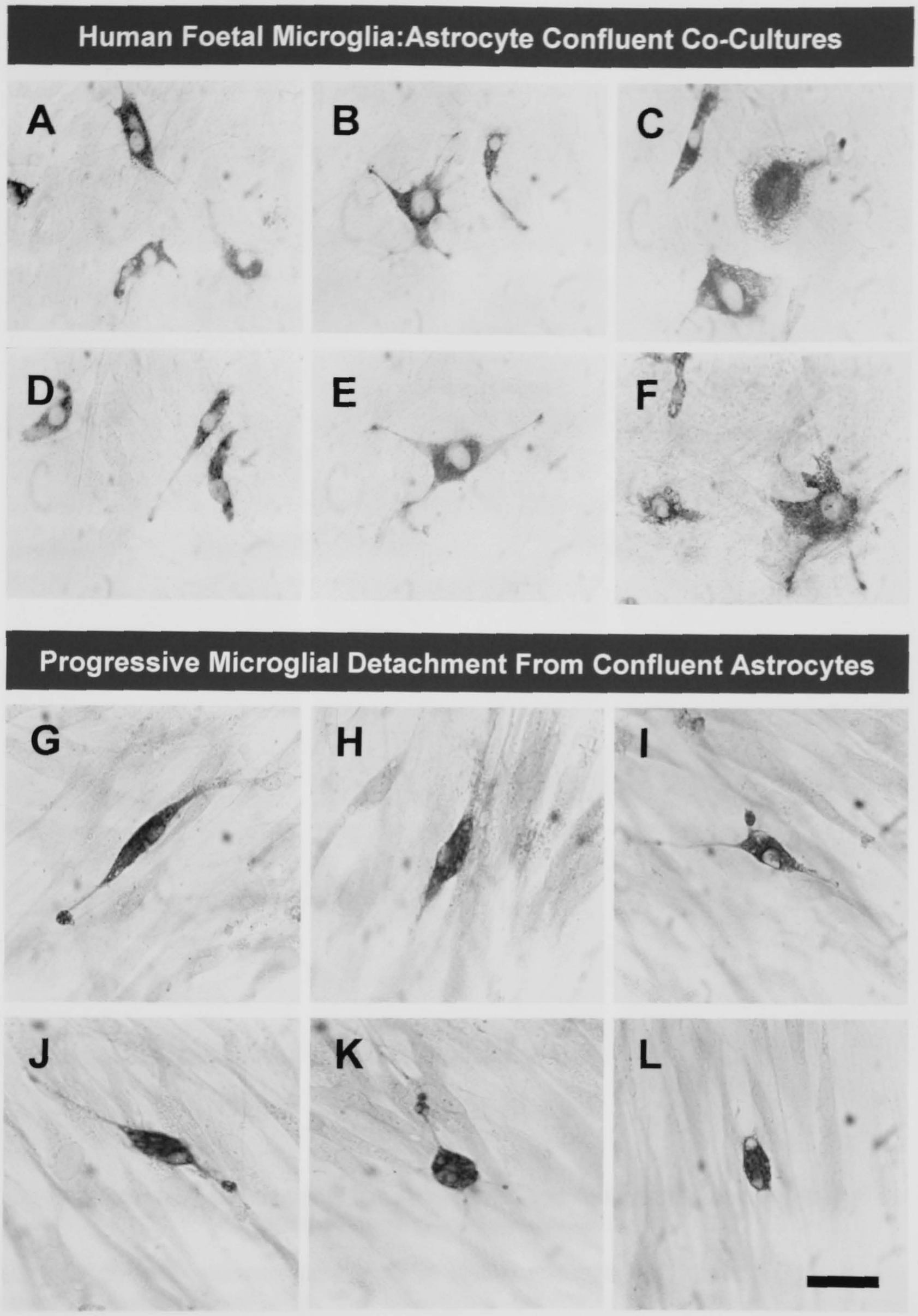


FIGURE 89

Morphology of human foetal microglia maintained in extended co-culture with confluent astrocytes

Light microscopic images of CD45:68 immunostained microglia in confluent astrocyte co-cultures. With prolonged culturing (>2-4 weeks), microglia progressively adopt bipolar, tripolar and flattened morphological forms below the confluent layer of astrocytes (**A-F**), whereas cells that overly the astrocyte cultures begin to retract their processes, round up and eventually detach from the astrocyte layer into the supernatant (**G-L**). Occasionally, cells that detach also undergo a form of controlled cell death (see Figure 90 and Figure 97).

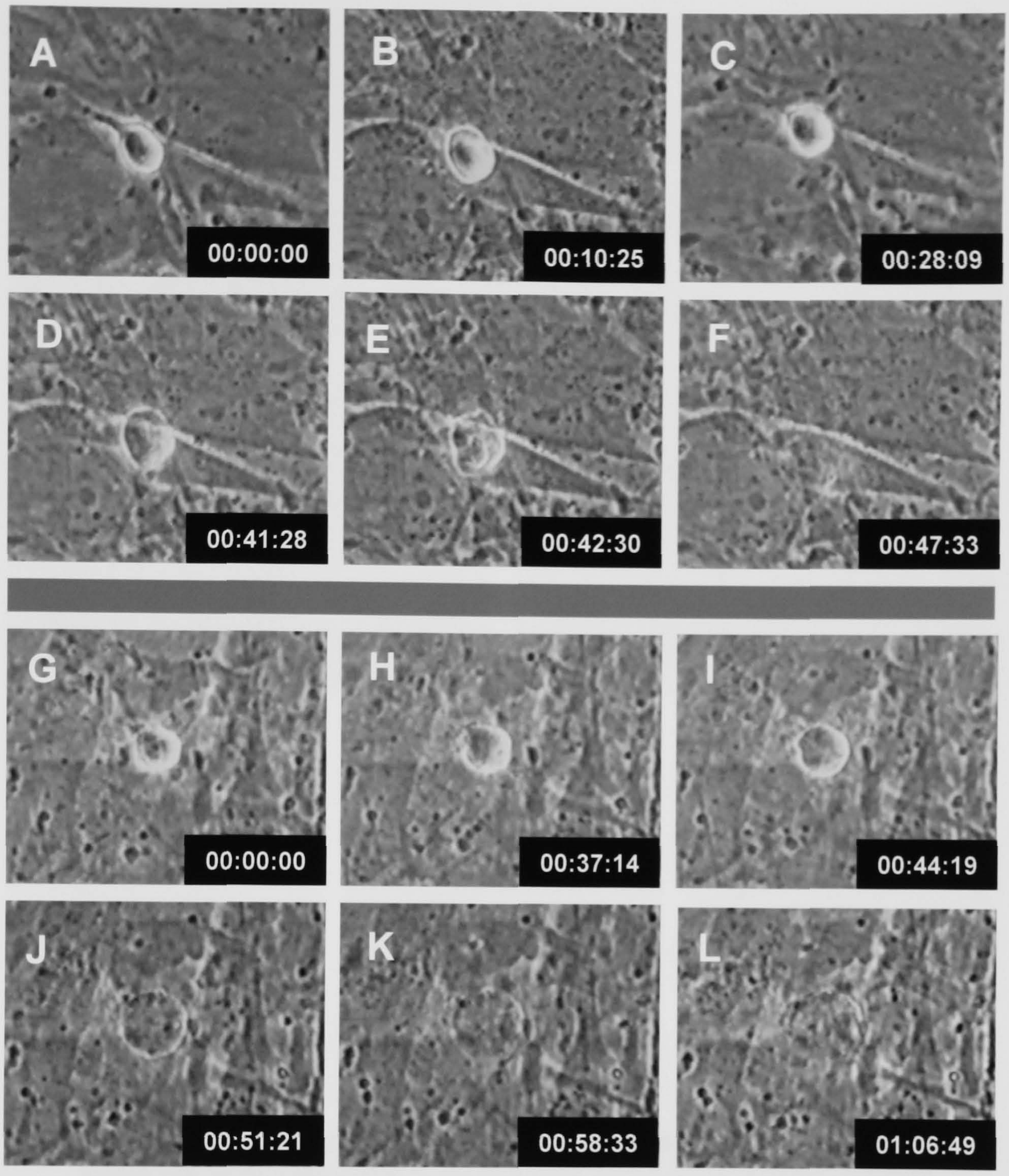


FIGURE 90

The process of microglial cell death following detachment from confluent astrocytes in co-culture preparations

Time lapse video microscopic images of solitary microglia (**A,G**) passing through the stages of detachment from the underlying confluent astrocytes and eventually undergoing a form of cell death which involves rapid cellular convulsions, blebbing at the cell surface, reorganisation of intracellular constituents, and rapid expansion. Note that the entire process was complete within an approximate period of 40 minutes to 1 hour, at the end of which the phase-dark cell remnant is still bound by a thin membrane at the periphery (**J-L**) (indicating that the cellular contents have been witheld from release, and that this may be form of programmed cell death, rather than a result of necrosis).

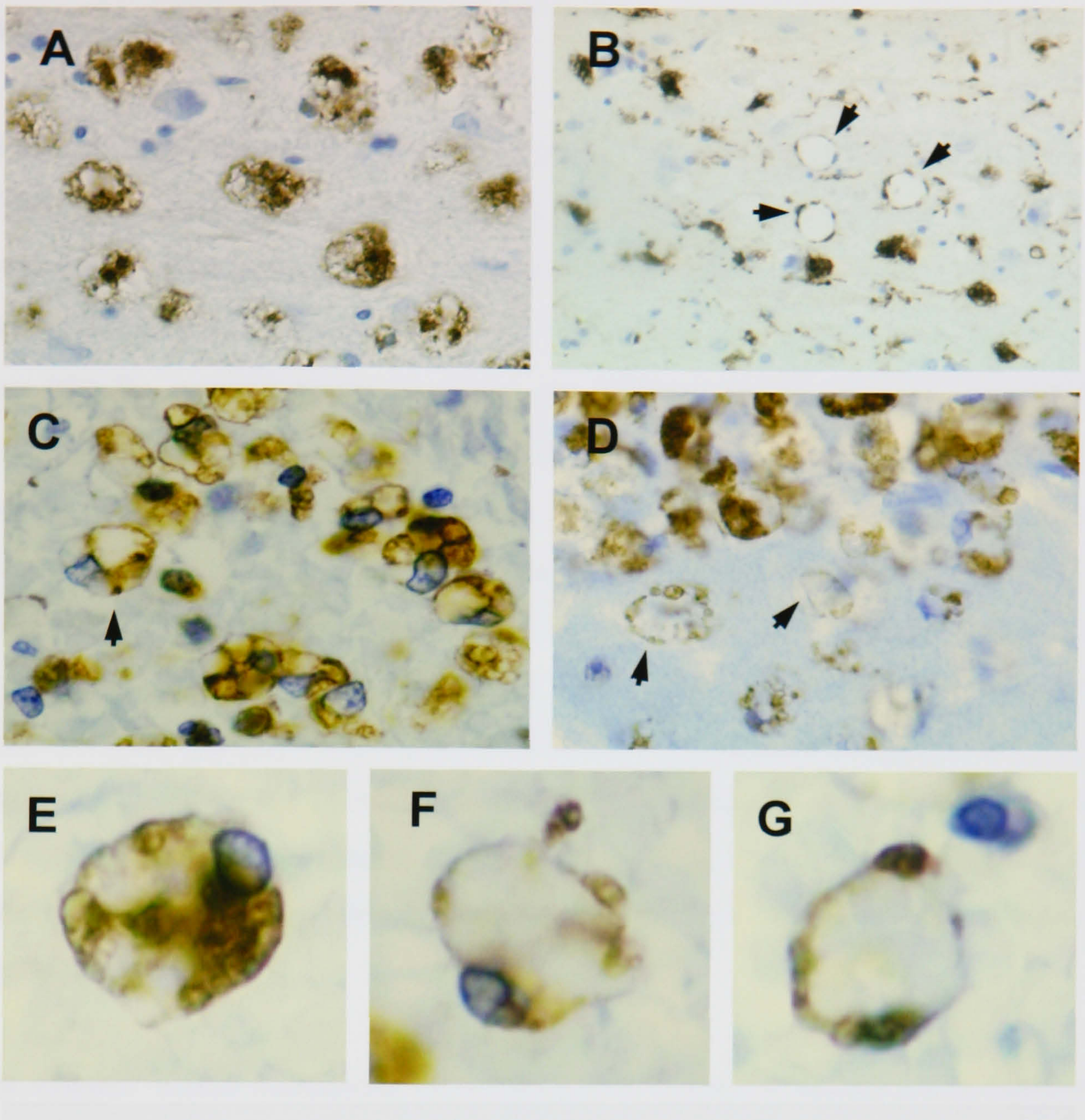


FIGURE 91

Macrophages within the CNS (A-B, E-G) in Creutzfeldt-Jakob disease and within the inner dura mater (C-D) of a case with sub-dural haemorrhage, presumed to undergo progressive cell death

Immunohistochemcal preparations showing various morphological forms of lipid-laden, vesicular, CD68 positive brain and dural macrophages, and their cellular remnants at the end-stage of disease. Note the weaker staining of membrane and vesicular components located at the periphery of defunct cells (arrows in **B, C, D**). **E-G**, show higher power examples of the proposed stages in the progression of macrophage cell death. Figures taken from previously unpublished work by Rezaie and Lantos (A,B,E-G) and Rezaie and Al-Sarraj (C,D).

Expression of chemokines and chemokine receptors by human foetal astrocytes and microglia in culture

Of all the chemokines tested in these culture preparations (MIP-1α, MIP-1β, MCP-1, MCP-3, RANTES, IL-8, IP-10, SDF-1), only MIP-1α and MCP-1 were reproducibly expressed under basal conditions (**Figure 92**, **Figure 93**). In human foetal astrocyte and microglial co-cultures, intense surface and cytoplasmic expression of MIP-1α was mainly restricted to microglia (**Figure 92A,B**). Isolated microglia expressing MIP-1α usually appeared in pairs (**Figure 92C**), a property that resembled MIP-1α positive microglia in the intermediate zone of the developing human telencephalon. Expression of this chemokine was also found at the surface of astrocytes and at borders of contact between astrocytes in longer-term confluent cultures (**Figure 93B,C**). MCP-1 was detected within clusters of sub-confluent foetal astrocytes (**Figure 93D**). However, immunoreactivity was variable between these non-stimulated cultures. Variable and weak expression of RANTES was also found, particularly associated with cell clusters (data not shown). Importantly, with progressive time, confluent astrocyte cultures showed MCP-1 expression at the surface and in distinct intense patches throughout cultures (**Figure 93E,F**), which corresponded closely with overlying intermediate-ramified microglia (**Figure 94**).

To test whether THP-1 monocytes would selectively adhere to human foetal astrocytes in a similar manner to that seen with microglia, and whether MCP-1 was important in regulating this interaction, isolated sub-confluent astrocytes were co-cultured with THP-1 cells for 24-48 hours. As can be seen from **Figure 95**, while many THP-1 cells aggregated on astrocytes that expressed MCP-1, this was not an exclusive finding, since a proportion of astrocytes clearly expressing this chemokine had very few or no adherent cells. Interestingly, a population of isolated THP-1 cells showed some degree of morphological differentiation into large microglia-like cells with bipolar or multipolar aspects and spiny or vellum-like processes when cultured for up to 48 hours in the presence of 50-100ng/ml recombinant human MCP-1 (data not shown). Taken together, these findings may suggest that MCP-1 and MIP-1α act as signals not only for the migration, but also the differentiation of microglia. Nevertheless it is likely that other factors (diffusible or non-diffusible) are additionally necessary to mediate the interactions between these cells. Clearly however, human foetal microglia did co-localise with areas of MCP-1 expression in cultures which attained confluency, as shown in **Figure 94**.

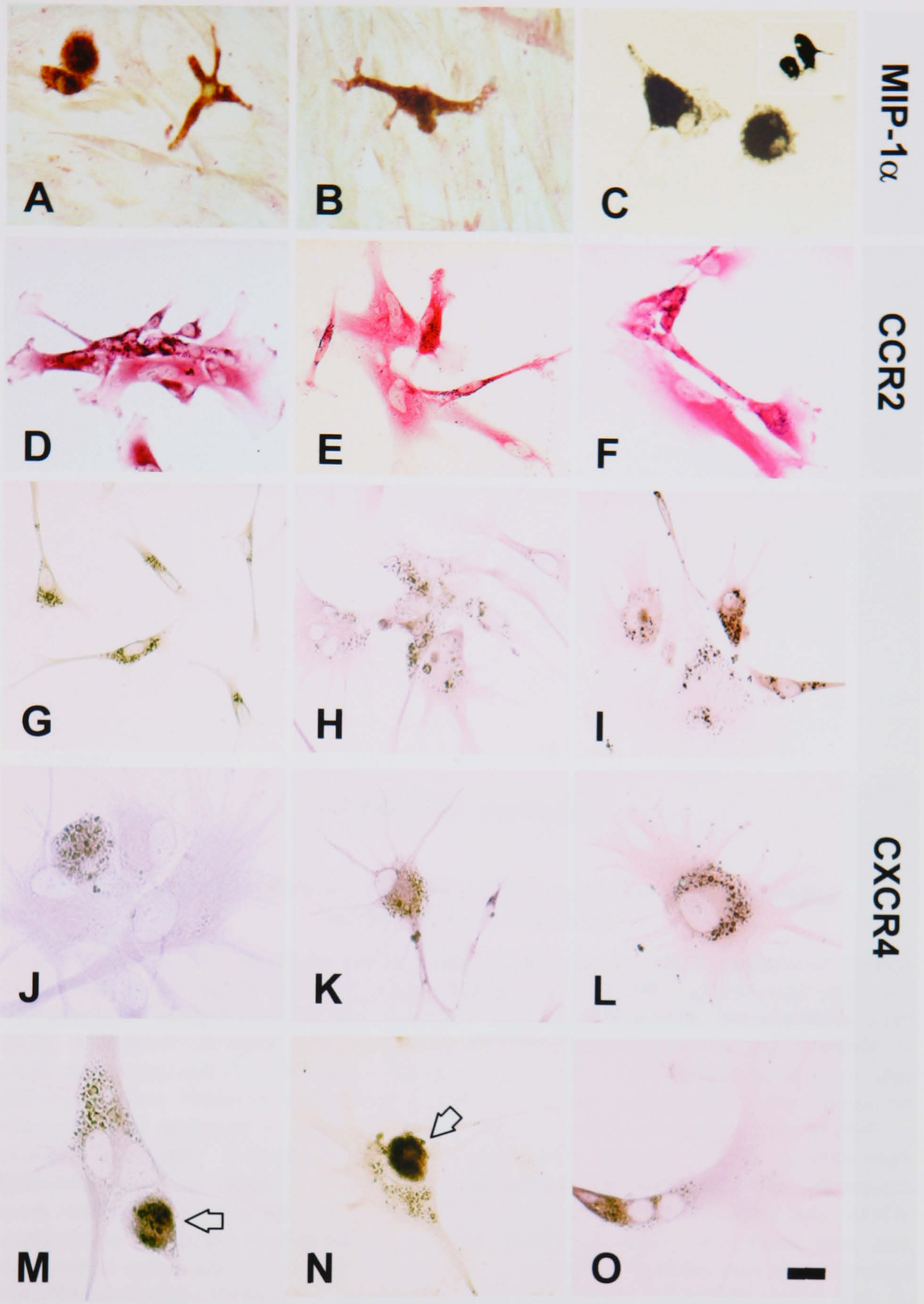


FIGURE 92

Expression of MIP-1α, CCR2 and CXCR4 in human foetal microglia and astrocyte co-cultures.

FIGURE 92 *(continued)*

Expression of MIP-1α, CCR2 and CXCR4 in human foetal microglia and astrocyte co-cultures.

Glial co-cultures derived from human foetal CNS between 13-19 gestational weeks, adopted a similar relationship with progressive time *in vitro*. Microglia in older confluent cultures were located both on the uppermost surface and beneath the astrocyte layer (**A,B**). Amoeboid microglia in these cultures demonstrated intense expression of MIP-1α (A,B, dark brown-black). Similarly, microglia in isolated culture preparations were also MIP-1α positive. However, expression of MIP-1α in these cultures was more intense on microglia that occurred in pairs (**C**, **inset**: higher magnification of a pair of MIP-1α positive microglia). Expression of the β-chemokine receptor CCR2 (purple-black granules) could be detected in GFAP-immunoreactive astrocytes and glial precursors (red) maintained in subconfluent cultures (**D-F**). Expression of this receptor was notably confined to vesicular compartments of glial precursors (**D,E**) and much less was detected in type I astrocytes (**E,F**). Dual-label immunocytochemistry also demonstrated CXCR4 expression (brown) by populations of GFAP-positive cells (pale violet) (**G-I**, **K-O**). Distinct vesicular and perinuclear expression of CXCR4 (brown) was detected in astrocytes (**G-I**, **K-N**), including type II (**K**) and type I (**L**) astrocytes, and in microglia overlying astrocytes (**J**). In addition to cytoplasmic expression, amoeboid microglia associated with astrocytes also expressed CXCR4 on their surface (**M,N arrows**). However, surface expression of CXCR4 was more limited in subconfluent astrocytes, with only a small proportion of GFAP positive cells (**O**) demonstrating CXCR4 at the surface. Cultures A,B, derived from CNS material at 15 gestational weeks, maintained for 25 days *in vitro*. C: 14 gestational weeks maintained for 32 days *in vitro*. D-F: 15 gestational weeks maintained for >30 days in culture, passaged once. G-I, K-O:19 gestational weeks maintained for 12 days in culture. L:19 gestational weeks maintained for 12 days in culture, passaged once (maintained for a total of 50 days *in vitro*). Immunoreactivity: A-C:MIP-1α, dark brown-black (DAB). D-F:CCR2, violet-black (Vector VIP)/ GFAP, red (Vector red). G-O: CXCR4, dark brown-black (DAB)/GFAP, violet (Vector VIP). Scale bar approx. 25μm in A,B,D-I, 20μm in C, 7μm in L, 14μm in K-O.

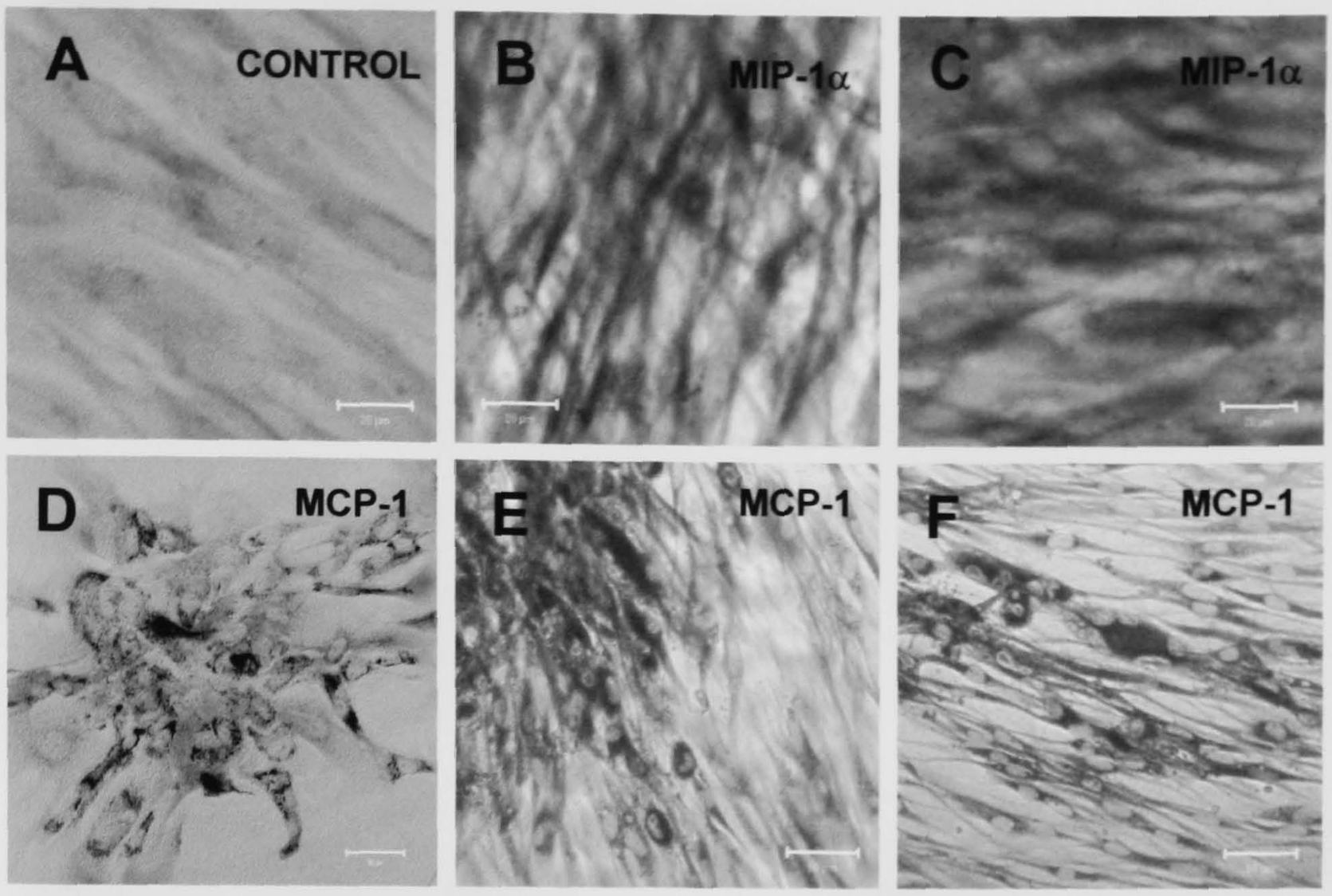


FIGURE 93

Expression of chemokines MIP-1α and MCP-1 on human foetal astrocytes.

Immunoreacivity to MIP-1α demonstrated expression of this chemokine on astrocytes (**B,C**) in confluent cultures and particularly at junctions between cells (**B**). In contrast, MCP-1 was expressed specifically within the cytoplasm of differentiating and dividing astrocytes in subconfluent cultures (**D**) and localised to distinct 'patches' of astrocytes in culture (**E,F**). These areas coincided with ramified microglia overlying progressively confluent astrocyte monolayers (see Figures 81, 89 and 94). (A) is a control culture where the primary antibodies to MIP-1α and MCP-1 were omitted from the immunocytochemical protocol. Scale bars represent approximately 20 μm in A-C and 50 μm in D-F.

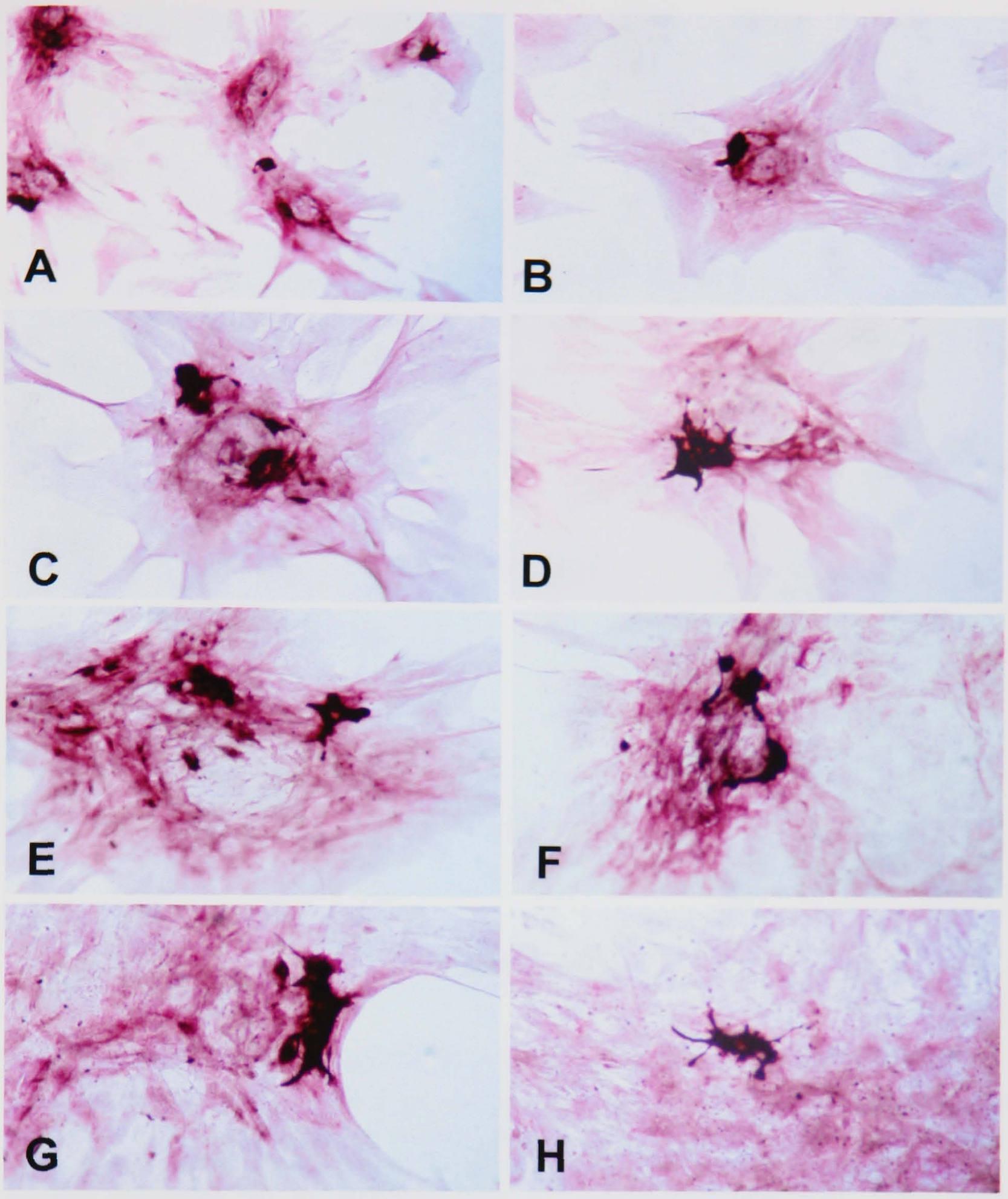


FIGURE 94

MCP-1 expression in human foetal microglia-astrocyte co-cultures

Immunocytochemistry with CD45:68 (blue-black: nickel-enhanced DAB), and MCP-1 (reddish-violet, VIP). Expression of MCP-1 occurs in distinct patches at the centre of aggregates of astrocytes in subconfluent cultures (**A-D**). With progressing confluence of cultures, MCP-1 is expressed more diffusely by astrocytes (**E-H**). At all stages, microglia (blue-black) are closely associated with areas of MCP-1 expression. Cultures prepared from samples at 14 gestational weeks.

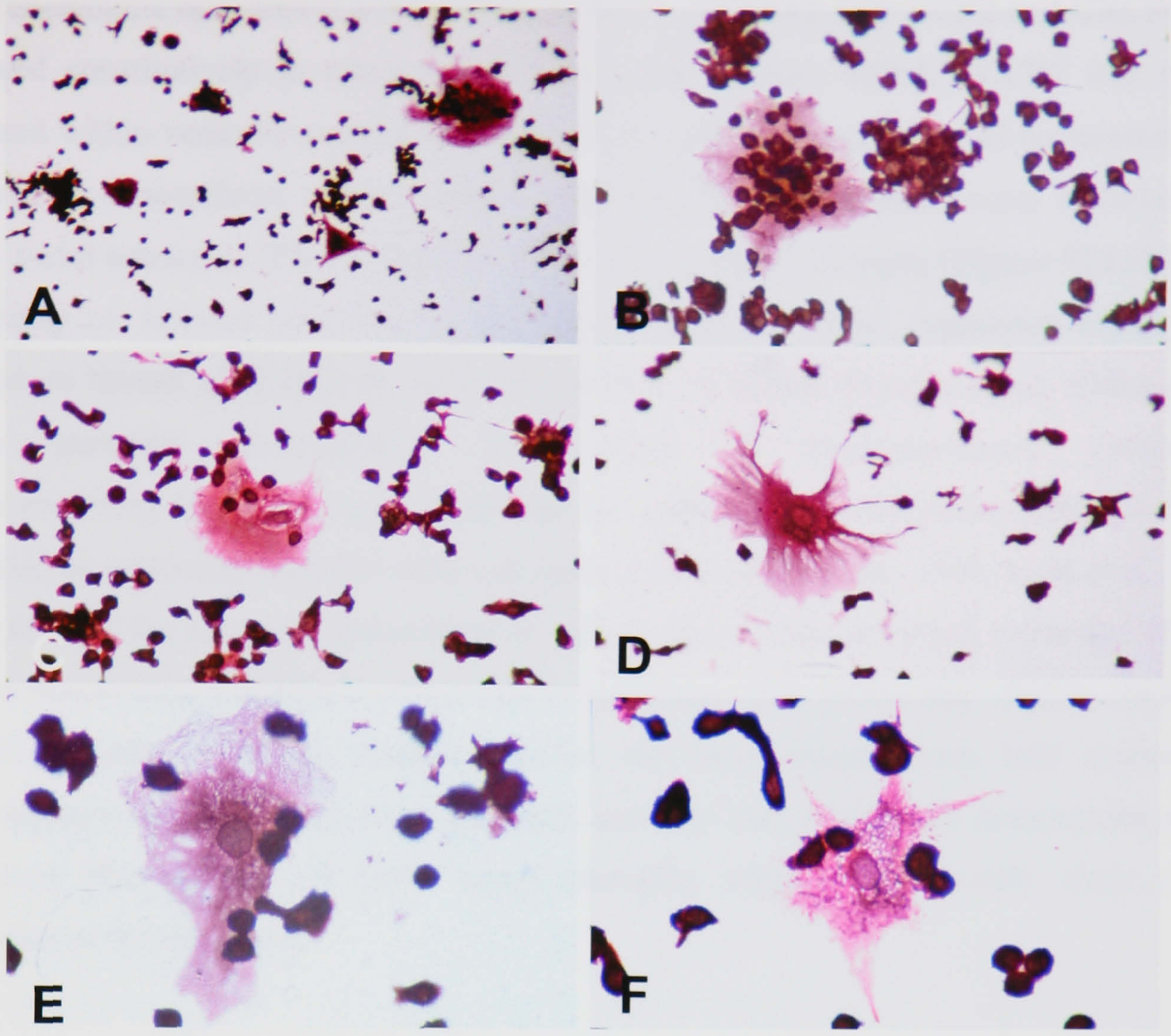


FIGURE 95

Interaction between human THP-1 monocytic cells and astrocytes *in vitro*

CD45:68 immunoreactive THP-1 monocytes (brown-black) cultured with human foetal astrocytes tend to aggregate on solitary astrocytes that expressed MCP-1 (red in A-D; violet in E,F) (**A,B**). Various stages in the interaction between THP-1 cells and MCP-1 positive astrocytes can be detected within 24 hours of co-culture (**C-F**). THP-1 cells typically adhere to the periphery of astrocytes, and are subsequently positioned at perinuclear sites on the surface of these cells. THP-1 cell proliferation is evident in some instances. However, it is clear from these figures that not all astrocytes expressing MCP-1 are bound, which signifies that additional factors are still required for monocytic adhesion.

Of the chemokine receptors (CCR2, CCR5, CXCR1, CXCR4), only CCR2 and CXCR4 were expressed constitutively in astrocytes and microglia (**Figure 92D-L**). CCR2 was notably expressed within vesicular compartments of clusters of glial precursors and occasional type I astrocytes in subconfluent cultures (**Figure 92D-F**). CXCR4 was much more widespread on human foetal astrocytes (**Figure 92G-I,K-O**), and associated microglia (**Figure 92J,M,N**) and specifically on isolated type I astroglia (**Figure 92H,I,L**). CCR5 expression although not detected on human glial cells *in vitro*, was present on human foetal neurons maintained in culture (personal observations). The pattern of intracytoplasmic juxtanuclear immunoreactivity for CCR2 and CXCR4 can be attributed to accumulation within the Golgi apparatus, as previously noted in other cell types (Halks-Miller et al., 1997; Lore et al., 1998). **Table 11** lists the estimated percentage of cells in these co-cultures that expressed CXCR4 under non-activated conditions (basal culture medium). This shows that slightly more than half of the cells in culture express CXCR4. Of these approximately half express low intracytoplamic levels (<10 granules per cell), and only 10% of the cells demonstrate surface expression (the majority of these being microglia, which predominantly show surface expression of this receptor).

Table 11. Expression of CXCR4 in microglia-astrocyte co-cultures

CXCR4 expression	Mean percentage of cells*
No expression	46.6
Low cytoplasmic levels	27.7
High cytoplasmic levels	25.7
Surface expression	10
Surface expression	6.17[a], 79.3[b], 14.5[c]

[*] Data presented as the mean percentage of cells examined in 100 fields (each field corresponding to an area of approximately 0.2mm^2) at x200 magnification, assessed in triplicate. Co-cultures were established from material obtained at 19GW.

[a] Out of the total number of astrocytes, 6.17% expressed CXCR4 at the surface

[b] Out of the total number of microglia, 79.3% expressed CXCR4 at the surface

[c] Of the total number of cells expressing CXCR4 at the surface, paired cells (microglia/astrocytes) represented 14.5%

Effect of recombinant human MCP-1 and MIP-1α on astrocyte proliferation

Addition of recombinant human chemokines to pure astrocyte cultures produced consistent and significant proliferation of these cells above control levels at 10ng/ml concentrations, as determined by nuclear counts (**Figure 96**). It is likely that at higher concentrations (>10ng/ml) receptors on these cells become saturated and proliferation is arrested or inhibited.

Effect of recombinant human chemokines and LPS on foetal CNS cultures

The effects of chemokines and LPS were next examined on MCP-1, CCR2 and CXCR4 expression in pure astrocyte cultures (**Table 12**), and in co-cultures derived from the CNS at 13,14 and 15 gestational weeks. Basal expression of MCP-1 was noted in clusters of cells in these subconfluent preparations (probably proliferating glial progenitors). Treatment of cultures with rhMCP-1 did not appreciably alter astrocytic MCP-1 expression in samples between 13 and 15 gestational weeks. Although rhMIP-1α induced expression of MCP-1 at [0.5-50ng/ml] in the 14-week sample, this is inconclusive as this observation could not be reproduced in samples derived from 13 and 15 gestational weeks. LPS at 50ng/ml induced moderate to widespread upregulation of MCP-1 expression in all glial cultures examined. The increased expression was related to widespread immunoreactivity in a greater population of cells (both astrocytes and microglia) in co-cultures.

Table 12. Effect of recombinant human chemokines and LPS on the expression of MCP-1 and CCR2 by human foetal astrocytes

MCP-1

	Unstimulated	LPS	rh MIP-1α	rh MCP-1
13GW	+	++ [50ng-0.5μg/ml]	+/-	+/-
14GW	+/-	+ [5-50ng/ml]	++ [0.5-50ng/ml]	+ [50ng/ml]
15GW	+/-	++ [5-50ng/ml]	+/-	+/-

CCR2

	Unstimulated	LPS	rh MIP-1α	rh MCP-1
13GW	+/-	+/-	+ [2.5ng/ml]	++ [50ng/ml]
14GW	+/-	+/-	+ [10ng/ml]	++ [50ng/ml]
15GW	+/-	+/-	+ [10ng/ml]	++ [50ng/ml]

[+/-] weak staining/no appreciable change in immunocytochemical expression
[+] mild-moderate immunoreactivity
[++] widespread and intense immunoreactivity

Effect of recombinant human chemokines MCP-1 and MIP-1α on human fetal astrocyte proliferation *in vitro*

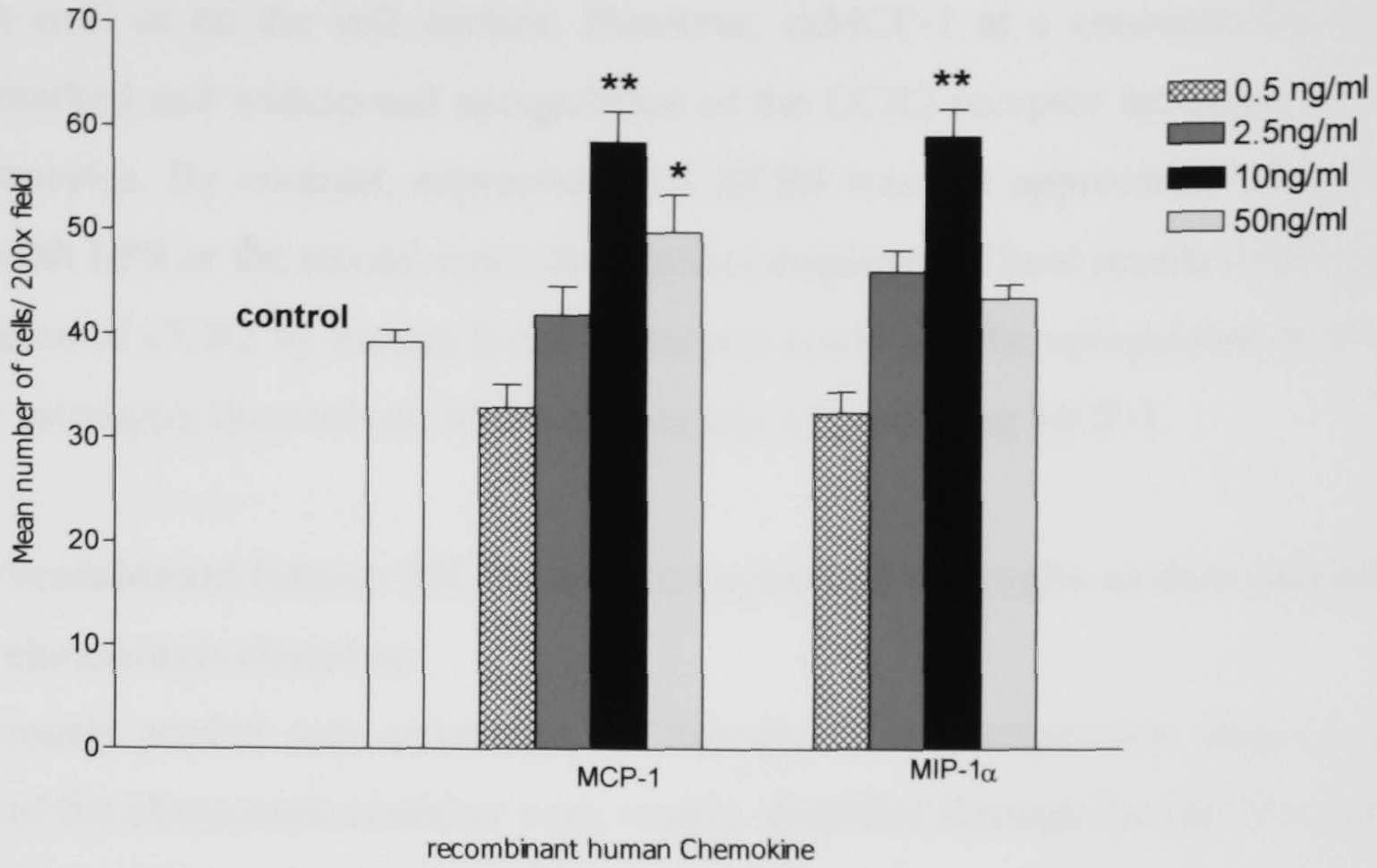


FIGURE 96

Effect of recombinant human chemokine treatment on the proliferation of human foetal astrocytes.

Human foetal astrocyte cultures were established from material derived at 19 gestational weeks, assayed in triplicate. Data represent the mean number of cell counts ± SEM, from 20 random fields in triplicate. Student's *t*-test: * very significant difference compared with controls ($p \geq 0.0$), ** highly significant difference compared with controls ($p \geq 0.001$).

The effects of chemokines on expression of the MCP-1 receptor CCR2 was also determined (**Table 12**, lower panel). Treatment of cultures for 18 hours with rhMIP-1α [2.5-10ng/ml] induced moderate upregulation of CCR2 receptors expressed intracytoplasmically within vesicles as well as on the cell surface. However, rhMCP-1 at a concentration of 50ng/ml produced marked and widespread upregulation of the CCR2 receptor including expression in Type I astrocytes. By contrast, expression of CXCR4 was not appreciably altered either by treatment with LPS or the recombinant chemokines employed. These results demonstrated that the expression of CCR2 by human foetal astrocytes could also be upregulated in an autocrine manner by astrocytes themselves, which are capable of producing MCP-1.

Effect of recombinant human MCP-1 on astrocytes and microglia as determined using the Dunn chemotaxis chamber

Cells previously seeded onto coverslips, or introduced as a suspension directly within the outer well of the chemotaxis chamber were readily identified through the use of calcein AM or Hoechst 33342 (**Figure 97**). When rhMCP-1 at [10ng/ml] was introduced within the inner well, cells in suspension (astrocytes and microglia) began to accumulate at the edge of the platform, after a period of exposure lasting between 2 and 6 hours (**Figure 97B**). Occasionally, one or two cells could be detected that were migrating at the edge of the platform towards the source of chemoattractant. However, the majority of those that had reached the platform were no longer migratory. Microglia co-cultured with astrocytes and seeded onto coverslips placed on the chamber, were identified with RCA-1 (shown in red). Their viability was assessed through their uptake and retention of calcein (in green), which showed that the dilution of lectin used (1:10,000) was tolerated by the microglia. By contrast, live microglia did not tolerate labelling with FITC-conjugated PG-M1 (CD68), even at very dilute concentrations (up to 1:5000) (data not shown). Nevertheless, examination of mixed glial co-cultures revealed that some of the cells showed signs of damage to DNA (fragmentation within the cytoplasm, unravelling and blebbing). This is shown more clearly in the higher power figures (**Figure 97F-H**), one of which is labelled with RCA-1 (**Figure 97H**).

The lower panel of the figure shows morphologies of individual RCA-1 labelled cells exposed to rhMCP-1. At [10ng/ml], rhMCP-1 induced reorientation of microglial cells, and produced polarised movement and rufflling of their membrane in the direction of the source of chemoattractant (to the right of figure 1f), within a one-hour period (**Figure 97, cell 1**). These cells were labelled with Hoechst 33342, RCA-1 lectin conjugated to rhodamine, and retained calcein AM which confirmed their viability (**Figure 97, cell 2, cell 3**). However, other than their polarisation and membrane ruffling, these microglia did not migrate any measurable distance in the direction of the chemoattractant. Instead they usually appeared to be in a state

of arrest after a period of around four hours. These phenomena could not be reproduced following exposure to rhMIP-1α under the conditions described.

When exposed to [100ng/ml] rhMCP-1 for a period of up to 6 hours, however, RCA-1 labelled cells began to alter their morphological presentation and progressively die (**Figure 97, cells 4-11**). In the initial phases, within 1-2 hours following exposure, labelled cells showed more diffuse labelling with Hoechst 33342, and in many instances the nucleus appeared distorted and distended. With longer exposures to this concentration of the chemokine, these cells clearly began to show signs of nuclear damage, resulting in fragmentation at terminal stages. Cells which were undergoing this form of cell death typically showed cytoplasmic convulsions (blebbing), with nuclei displaced to the periphery, prior to defragmentation. Once again, these alterations could not be demonstrated with rhMIP-1α or IL-8 under the stipulated conditions. These results indicated that human foetal microglia were capable of responding to MCP-1 at physiological levels, by reorientation and cytoskeletal changes such as membrane ruffling, whereas at higher concentrations, MCP-1 actually induced cell death under these experimental conditions. Intriguingly, the initial experiments with cell suspensions also suggested that astrocytes as well as microglia could respond to MCP-1 (as indicated by the accumulation of cells at the edge of the platform). However, this was not pursued further during the course of this investigation, and will require further critical assessment.

FIGURE 97

Fluorescent microscopic images of mixed glial cells (A-H) and lectin labelled microglia (1-11) subjected to various concentrations of recombinant human MCP-1 and assayed in the Dunn Chemotaxis Chamber

FIGURE 97 *(continued)*

Fluorescent microscopic images of mixed glial cells (A-H) and lectin labelled microglia (1-11) subjected to various concentrations of recombinant human MCP-1 and assayed in the Dunn Chemotaxis Chamber

(**A**) Pale Hoechst 33342-fluorescent cells can be identified on the platform of the chemotaxis chamber, located under a glass coverslip previously seeded with isolated cells. (**B**) A cell suspension has been placed within the outer well of the chemotaxis chamber, and [10ng/ml] rhMCP-1 located within the inner well. After a period of exposure lasting between 2 and 6 hours, cells began to accumulate at the edge of the platform, and occasionally, one or two cells could be detected that had reached the summit and were positioned on the edge of the platform, directed towards the source of chemoattractant. (**C-E**) Microglia co-cultured with astrocytes and seeded onto coverslips placed on the chamber, could be readily detected with RCA-1 (shown in red), and these were viable through their uptake of calcein (in green). However, examination of glial co-cultures revealed that some cells showed signs of damage to DNA (fragmentation within the cytoplasm, unravelling and blebbing). This is shown more clearly in higher power figures (**F-H**). An RCA-1 labelled microglial cell is shown in (**H**), with an abnormal nucleus which has begun to fragment.

The lower panel shows morphology of individual RCA-1 labelled cells exposed to rhMCP-1. (**1a-f**) At [10ng/ml] rhMCP-1 induced reorientation of the microglial cell as shown, and produced polarised movement and ruffling of its membrane in the direction of the source of chemoattractant (to the right of figure 1f), within a one-hour period. (**2a-c**) demonstrates one such cell labelled with Hoechst 33342 (**2a**), RCA-1 lectin conjugated to rhodamine (**2b**), and calcein AM to show cell viability (**2c**). A second example is shown in (**3a-b**), after a period of exposure for 2 hours. Figures (**4-11**) are examples of individual cells exposed to [100ng/ml] rhMCP-1 for a period of up to 6 hours. In the initial phases (1-2 hours) following exposure, labelled cells showed more diffuse labelling with Hoechst 33342, and in many instances the nucleus appeared distorted and distended (**4-6**). With longer exposures to the chemokine, these cells clearly began to show signs of nuclear damage, and at terminal stages, resulting in fragmentation (**10-11**). Cells which were undergoing this form of cell death typically showed cytoplasmic convulsions (blebbing), with nuclei displaced to the periphery, prior to defragmentation.

Chapter V GENERAL DISCUSSION

Microglia: their origins and relationship with the mononuclear phagocyte system

We saw in Chapter I, that monocytes and macrophages were originally classified as cells of the *'reticulo-endothelial system'* by Aschoff in 1924. van Furth and colleagues (1972) introduced the concept of a *'mononuclear phagocyte system'* to further define the system of monocytes and macrophages. Macrophages, by definition, are any of the diverse forms of mononuclear phagocytes found in tissues (Andreesen 1990, 1991, 1992; Dijkstra and Damoiseaux, 1993; Gordon, 1988; Michna 1988; Zwilling and Eisenstein, 1993). It was generally held that the development of these cells took place within the bone marrow and passed through the following stages: (i) *within the bone marrow*- stem cell, committed stem cell, monoblast, promonocyte, monocyte, (ii) *within peripheral blood*- monocytes which circulate for up to two days, (iii) *within tissues*- resident macrophages. The differentiation of monocytes within the bone marrow proceeds fairly rapidly (1.5-3 days) during which cytoplasmic granules are found in monocytes (consisting of non-specific granules containing lysosomal enzymes and some lysozyme, and azurophilic granules which appear violet with Wright's stain). Following emigration into tissues, monocytes transform into macrophages. The morphology of these cells varies among different tissues and between normal and pathological states. Most possess a round or indented nucleus, a well-developed Golgi apparatus, abundant endocytic vacuoles, lysosomes and phagolysosomes (which they can regenerate) and a ruffled plasma membrane covered with microvilli. Several recent articles have highlighted the various aspects of the biology of macrophages (Andreesen 1990, 1991, 1992; Dijkstra and Damoiseaux, 1993; Gordon, 1988; Michna 1988; Zwilling and Eisenstein, 1993). We shall briefly examine the heterogeneity and activation of these cells, outline some of their important functions, and address their rate of turnover, before relating these findings to the CNS.

Heterogeneity and activation of macrophages

The heterogeneity of macrophages is well documented. It has been recognised for some time that macrophages isolated from various tissues display diverse phenotypes and functional capabilities. Their functions in particular, are related to signals received in their immediate environments, unique to different tissues. For example, the anaerobic environment and cellular composition of the spleen or peritoneum confers different properties on resident macrophages than the aerobic environment of the alveolar macrophage: as a result, although lung macrophages are proficient in performing functions that include phagocytosis and secretion of nitric oxide, they are somewhat deficient at others such as antigen presentation, when

compared with macrophages found within the spleen or peritoneum. The phenotype of macrophages, that is, their expression of specific membrane antigens, is usually a reliable indicator of their activational states, and related to pathophysiological functions. For this reason, antibodies directed against specific membrane antigens have been used to compare macrophages from different tissues.

Generally speaking, resident tissue macrophages are quiescent immunologically, with low levels of oxygen consumption, low constitutive levels of MHC class II gene expression, and little or no cytokine secretion. Nevertheless, they retain the ability for chemotaxis and phagocytosis, and the capacity to proliferate. There are two stages to macrophage activation: the first being a '*primed*' state, in which these cells upregulate expression of MHC class II expression, antigen presentation and oxygen consumption, but retain a reduced proliferative capacity. Interferons, IL-3, GM-CSF and TNF-α can prime macrophages for selected functions. Once primed, macrophages can respond to further stimuli to become fully activated, a stage defined by their ability to proliferate, their high oxygen consumption (through NADPH oxidase), killing of parasites, tumour cell lysis and maximal secretion of inflammatory mediators (TNF-α, PGE_2, IL-1, IL-6, reactive oxygen species and nitric oxide via inducible nitric oxide synthase). Bacterial LPS, yeast glucans, GM-CSF and phorbol esters are amongst the factors that may provide secondary signals. Nevertheless, a clear distinction between primed and fully activated macrophages is usually arbitrary, and depends largely on the stimulus and function assessed. Although macrophages may be primed relatively easily in response to stimuli, considerably more activation is required to achieve the cytotoxic 'fully-activated' state. Macrophage activation may therefore be viewed as a multi-step process.

Functions and turnover of macrophages

The functions of macrophages include chemotaxis, non-specific phagocytosis and pinocytosis, specific phagocytosis of opsonised micro-organisms via Fc and complement receptors, proteolytic digestion, processing and presentation of antigens to T and B lymphocytes and the secretion of a plethora of products including enzymes (matrix metalloproteinases, lysozyme, collagenases, elastase and hydrolases), complement components and coagulation factors, prostaglandins and leukotrienes and several regulatory cytokines, chemokines and growth factors (including PDGF and bFGF). Macrophages are extremely sensitive to hypoxia and are triggered to proliferate and signal the recruitment of other mononuclear cells. Inflammation triggers resident macrophages to scavenge and eliminate dead tissue. Macrophages also play important roles in wound repair and healing, and in angiogenesis. In the process of clearance, macrophages secrete by-products that stimulate matrix-producing cells to lay down complexes and scar tissue. Products secreted by macrophages also stimulate the growth of new blood

vessels, in order to maintain a supply to newly formed tissues. Subsequently, macrophages are additionally involved in refining and remodelling the scar, in order to further strengthen and support the repaired tissue. This reorganisation within tissues, is brought about by enzymes secreted by macrophages, which include hyaluronidase, elastase, collagenase, matrix metalloproteinases as well as inhibitors of some of these (antiproteases), regulatory growth factors, and so forth. As specific examples, hyaluronidase, through degradation of hyaluronic acid (a significant component of the extracellular matrix-ECM), reduces viscosity and permits greater spreading of material within tissue spaces. In a similar fashion, elastase and collagenase degrade elastin and collagen, which are basic components of the ECM. **Table 13** lists some of the effector and regulatory products of macrophages that have been characterised.

Blood monocytes already possess the capacity for migration, chemotaxis, pinocytosis and phagocytosis, functions attributed to macrophages and other mononuclear phagocytes, and express receptors for IgG Fc domains (FcγR) and complement receptors (e.g. C3bi component). Following migration into tissues, monocytes undergo further differentiation within a period of 48 hours to become multifunctional tissue macrophages. Under normal conditions, the resident population of tissue macrophages can be maintained by the influx of monocytes from the circulating blood or by local proliferation. At *steady state*, the renewal of tissue macrophages occurs mainly through local proliferation of progenitor cells and not via influx of monocytes. Although it was originally considered that tissue macrophages were long-lived cells, this is not a general rule. The longevity of a macrophage will generally depend on the tissue in which it resides. More recently, depending on tissue type, estimates of their viability is thought to range from one week to several months. For example, splenic macrophages circulate into and leave the spleen regularly: the turnover of alveolar macrophages is rapid (within days) due to the high exposure to particulate matter; Kupffer cells may remain in the liver for months or more, and the lifespan of microglia is speculated to even exceed that for Kupffer cells, with proliferation *in situ* as the main source for supplementing the microglial population.

Table 13. Effector and regulatory products of macrophages

Microbicidal and cytotoxic	
reactive oxygen intermediates	superoxide, hydrogen peroxide, hydroxyl radical, chloramines
reactive nitrogen intermediates	nitric oxide, nitrites, nitrates
oxygen independent	neutral proteases, acid hydrolases, lysozyme, defensins
Tumouricidal	hydrogen peroxide, nitric oxide, TNF-α, C3a, proteases, arginase, thymidine
Tissue destructive	hydrogen peroxide, TNF-α, nitric oxide, neutral proteases
Fever inducing	
pyrogenic cytokines	IL-1, TNF-α, IL-6
Inflammatory regulators	
bioactive lipids	prostaglandins (PGE_2,$PGF_{2\alpha}$), prostacyclin (PGI_2), thromboxanes, leukotrienes (LTB_4, LTC_4, LTD_4, LTE_4)
bioactive oligopeptides	glutathione
complement components	C1, C2, C3, C4, C5, factors B, D, P, I H
clotting factors	V, VII, IX, X, prothrombin, plasminogen activator inhibitors
cytokines	IL-1, IL-6, IL-8, IL-10, TNF-α, IFN-α/β, macrophage inflammatory proteins (MIP-1, MIP-2, MIP-3), regulatory growth factors (MCSF, GMCSF, GCSF, PDGF)
neutral proteinases	elastase, collagenase, angiotensin convertase, stromelysin
protease inhibitors	α2-macroglobulin, α1-proteinase inhibitor, plasmin and collagenase inhibitors, plasminogen activator inhibitors
acid hydrolases	acid proteases (cathepsin D and L), peptidases, lipases, lysozyme and other glycosidases, ribonucleases, phosphatases, sulphatases
stress proteins	heat shock proteins
Tissue remodelling	elastase, collagenase, hyaluronidase, matrix metalloproteinases, regulatory growth factors, fibrobalst growth factor (FGF), transforming growth factors (TGF-α, TGF-β), angiogenic factors
Miscellaneous	Apolipoprotein E (apo-E), IL-1 inhibitors, purine and pyrimidine derivatives (thymidine, uracil, neopterin)

Mononuclear phagocytes of the CNS

There are at least four anatomically (and most likely functionally) distinct mononuclear phagocyte populations associated with the CNS, apart from microglia, which reside within the CNS parenchyma (Perry 1997; Perry and Gordon 1991, 1997). They include meningeal macrophages, supraependymal macrophages at the CSF-ependyma border, macrophages of the choroid plexus (Kolmer cells), and perivascular macrophages (or 'perivascular cells') that are bounded by a basement membrane, lying within blood vessels. This latter population of perivascular macrophages has emerged as a group of cells that are located outside the CNS parenchyma proper (Graeber et al. 1989, 1990). In this respect, they differ from perivascular microglia that lie close to vessels within the parenchyma, and from pericytes within vessel walls that are believed to derive from smooth muscle precursors **Figure 98**. Perivascular cells are long spindle-shaped, or flattened ovoid cells lacking direct contact with neural cellular elements. They constitutively express several myelomonocytic antigens that are absent from

resting microglia. They express MHC class II antigens and are capable of synthesising T cell co-stimulatory molecules such as B-7.

Are microglia a component of the mononuclear phagocyte system?

There are several lines of evidence (including phenotypic and functional characteristics) to support the concept that microglia belong to the system of mononuclear phagocytes (Aloisi; 2001, Berry and Butt, 1996; Bruce-Keller, 1999; Gehrmann et al. 1995; Kreutzberg et al. 1996; Ling et al. 2001; Stoll and Jander, 1995; Streit, 1995; Streit et al. 1988). Activated microglia have a high content of hydrolytic enzymes (Ling and Wong, 1993; Williams et al. 1994). They behave in much the same manner as other tissue macrophages, and their activation is characterised by transformation into motile phagocytic cells that secrete proteases, cytokines, reactive oxygen species and nitric oxide. They are capable of phagocytosing dead or dying neurons (neuronophagia) and of 'stripping' synapses from certain neuronal populations (e.g. facial motor neurons), whereby they interpose processes between afferent synaptic terminals and the neuronal surface. The reorganisation (stripping) of synaptic inputs to injured neurons contributes towards aiding recovery (Blinzinger and Kreutzberg, 1968). *In vitro*, their phagocytic activity has been associated with expression of receptors for complement and Fc receptors for IgG (Ulvestad et al. 1994). Furthermore, cellular activation and morphological transformation of foetal microglia can be stimulated by factors known to influence and activate macrophages including lipopolysaccharide (Hetier et al. 1988; Suzumura et al. 1991), colony-stimulating factors (Sawada et al. 1990; Suzumura et al. 1990), and IFN-γ (Frei et al. 1987; Suzumura et al. 1987).

Activation of microglia is also associated with a marked upregulation of surface markers including MHC class II antigen, CD11b, CD64 and CD68. Microglia-derived TNF-α can produce bystander damage to oligodendrocytes during demyelination. Free oxygen radicals released by microglia exert neurotoxic effects in co-cultures of neurons and microglia. These cytotoxic properties of microglia can be modulated by cytokines and neurotransmitters. For example, cytokines such as IFN-γ can prime microglia to become activated. Others like TGF-β or IL-4 downmodulate the cytotoxic and phagocytic potential of microglia. Noradrenaline has been shown to reduce IL-1 and free radical production in macrophages and β-adrenergic agonists (e.g. isoproterenol) can reduce IL-1, and TNF-α production by microglia.

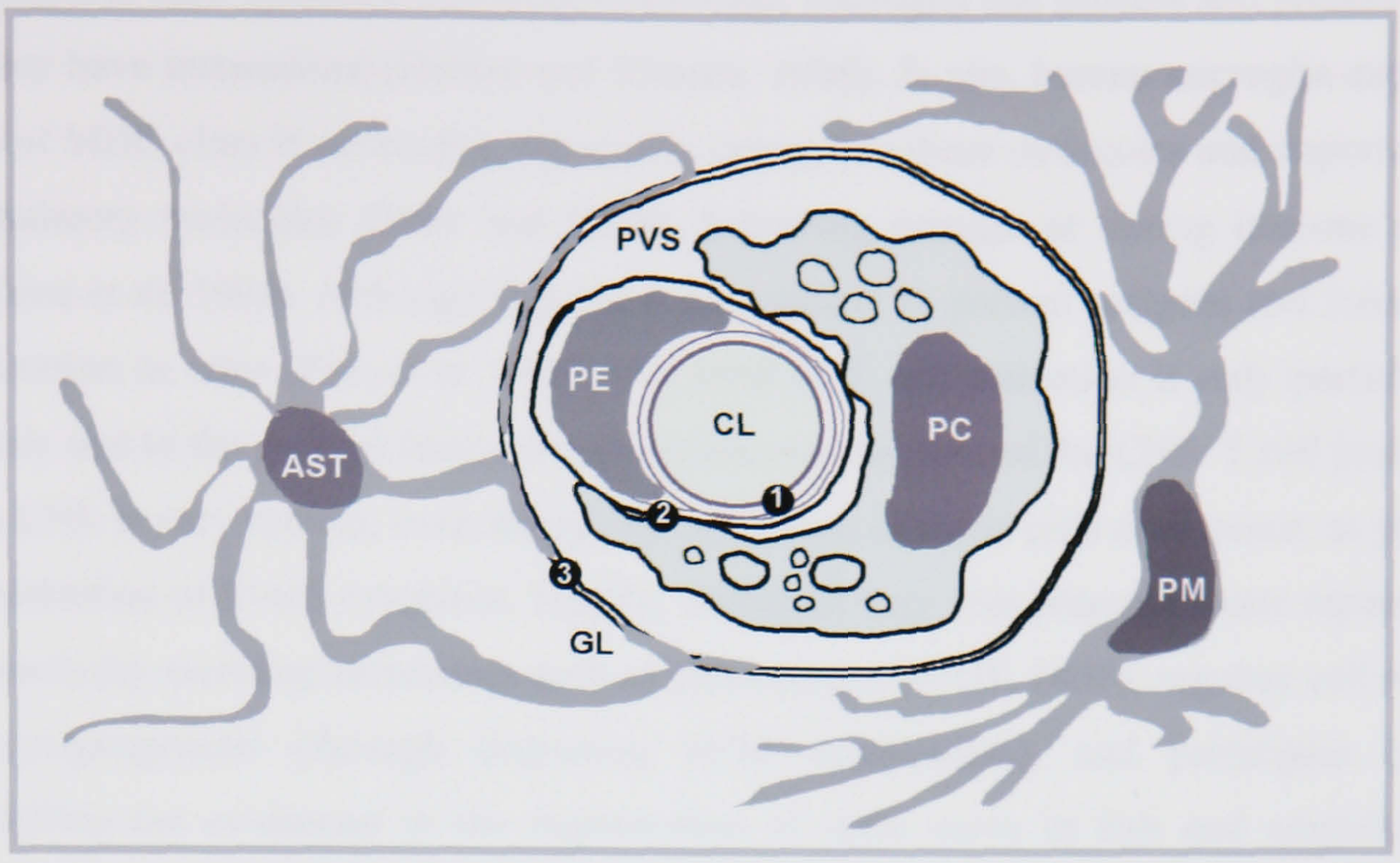


FIGURE 98

Schematic illustration to show the distinction between perivascular cell types: pericytes, perivascular cells and perivascular (parenchymal) microglia

Note the anatomical distinctions between pericyte (**PE**), perivascular cells (**PC**) (macrophages) and perivascular (CNS parenchymal) microglia (**PM**). Annotations: **[1]**,**[2]**,**[3]** represent the capillary, pericytal, and glia limitans basal laminae respectively; **GL**: glial limitans; **CL**: capillary lumen, **PVS**: perivascular space; **AST**: astrocyte.

In addition to their cytotoxic and phagocytic roles, microglia can process and present antigens that they have internalised (Hickey and Kimura, 1988). *In situ*, human microglia express low levels of MHC class II molecules, but widely upregulate these molecules and important T cell costimulatory molecules CD80 and CD86, following damage or during immune reactions (Williams et al. 1994). Although they have the capacity to present antigens and induce T cell proliferation *in vitro* (Frei et al. 1987), the level of T cell activation is only partial *in vivo*, probably due to the general immuno-suppressive environment of the CNS. T cell proliferation in the CNS is very limited, even though the activation of these cells does occur, as judged by the production of T-cell cytokines. Finally, microglia may also promote tissue repair, neurite outgrowth (by secreting substances such as plasminogen, bFGF, NGF), regulate cell migration and synaptogenesis (through degrading ECM components), and participate in tissue remodelling (as evidenced in the regeneration of optic nerve in fish and amphibians and segments of the spinal cord in rodents). As a result, microglial activation, like that of macrophages, has been considered as a 'double-edged' response.

Nevertheless, a property that sets microglia apart from other mononuclear phagocytes is their expression of an inward-rectifying potassium channel, and lack of outward current channels (Kettenmann et al. 1993; McLarnon et al. 1997). This property, which is retained across species (rodent and human), renders microglia very sensitive to depolarising events, since relatively small changes in membrane potentials trigger large inward currents, a phenomenon that is directly related to their capability for a rapid response to pathological stimuli. Microglial ion channels may be linked to P2 purinoceptors that become activated by ATP released from damaged cells. Microglial activation may therefore be triggered by pronounced depolarisation in response to tissue damage, during normal wear and tear, and after traumatic or ischaemic injury. Microglia share this inward rectifying channel with a population of precursor cells within the bone marrow, a finding that has direct bearing on the possibility of microglial progenitors being derived from the bone marrow.

Where do microglia originate from?

Developmentally, microglia have been considered to derive principally from four separate sources: (i) bone marrow progenitors, circulating in the blood, (ii) intrinsic bipotential mesodermal progenitors within the nervous system, seeded early during embryonic development, (iii) mesenchymal progenitors located around the nervous system, which penetrate the brain parenchyma, (iv) from neuroectodermal progenitors in the ventricular zone that give rise to glial and neuronal cell populations. The meningeal (pial) origin for microglia first proposed by del Rio-Hortega, has received support from several studies (Boya et al. 1979; Cammermeyer 1070; Dougherty 1944). In the last decade, Boya and colleagues (1991)

reported that meningeal macrophages (of mesodermal origin) invade the brain parenchyma and transform into ramified microglia. However, other authors have held the contrary opinion, that microglia like astrocytes and oligodendrocytes, arise from neuroectoderm-derived glioblastic progenitors (Fujita et al. 1981; Hao et al. 1991; Kurtz and Christ, 1998; Lewis 1968; Metz and Spatz 1924; Richardson et al. 1993; Pruijs 1927; Rydberg 1932: Schaffer 1926; Schelper and Adrian 1986). Alliot and co-workers (1991) postulated that the central nervous system contains and intrinsic pool of pluripotent haematopoietic-related stem cells, whereas yet others have hypothesised dual lineage and time courses for microglial development (Chugani et al. 1991; Eglitis and Mezey, 1997).

Although these alternative hypotheses cannot be entirely dismissed, evidence accumulated over the past two decades, has placed microglia within the unifying hypothesis of the mononuclear phagocyte system of van Furth and colleagues. The majority of these independent studies have offered support for the bone-marrow progenitor hypothesis (Ling and Wong, 1993). The reasons for this are as follows. In the 1980s, it was confirmed mainly through immunohistochemical studies, that microglia share epitopes with blood monocytes, lymphocytes and tissue macrophages, which are not present on astrocytes or oligodendrocytes. Prior to this, initial experiments with carbon-labelled blood monocytes prepared from adult donor Lewis rats, showed these cells to be subsequently located in the corpus callosum of postnatal recipient animals, following injection into the circulation of the recipient animals. The labelled cells progressively transformed to amoeboid cells (Ling and Tan, 1974). Other studies using transfected cells, bone marrow chimaeras and transplantation experiments have further shown cells which are derived from the bone marrow to enter the newborn and adult CNS in rodents, where they take up residence predominantly around blood vessels (in the perivascular space), as well as within the choroid plexus and the meninges (DeGroot et al., 1992; Flügel et al. 2001; Hickey and Kimura, 1988; Hickey et al. 1992; Krall et al. 1994).

Mononuclear phagocytes within the meninges and perivascular macrophages in particular, possess a relatively rapid turnover, and within a few months, large numbers of donor cells are located in these compartments (Hickey et al. 1992). By contrast, adult 'resting' microglia bearing donor markers are extremely rare. This evidence, together with the observation that there is no perivascular infiltration of mononuclear cells in rodents following transection of facial motor neurons (Graeber et al. 1988) suggested that the intrinsic adult microglia have a very low turnover in comparison with perivascular cells (Perry and Gordon, 1991). From their studies, using immunohistochemistry combined with autoradiography in mice, Lawson and colleagues (1992) came to the conclusion that the microglial population is maintained through slow turnover by an equal share of division of resident cells *in situ*, and by infiltration of

monocytes. They estimated that it would take the lifetime of the mouse for the entire microglial population in the steady state to be replaced by immigration of monocytes alone. In a separate investigation, Yeager et al. (1992) noted that after 8 months in the mouse. many of the host cells were of donor origin. Therefore, in the adult, it is mainly the perivascular cell population, rather than parenchymal microglia, which are continually renewed from circulating bone marrow precursors. Indeed, the aforementioned bone marrow chimaera studies have shown resident microglia to have a very low turnover with bone marrow precursor cells (less than 1%), whereas the percentage of macrophages is considerably higher in the leptomeninges (60%), and for perivascular cells (30%) (DeGroot et al. 1992; Flügel et al. 2001; Hickey and Kimura, 1988; Hickey et al. 1992: Krall et al. 1994).

The only comparative studies of the renewal of microglia in humans have emerged following bone marrow transplantation (Krivit et al. 1995; Unger et al. 1993). Unger and colleagues (1993) were able to histologically identify the Y-chromosome in microglia within the CNS of female patients who had received bone marrow transplants from male donors. Their study demonstrated enhanced immunoreactivity predominantly for perivascular microglia with an antibody to CD45, without perturbance of the blood-brain barrier. These authors were able to detect CD45/Y-chromosome positive cells in the medulla and inferior olive of five females who had received male bone marrow transplants. Krivit and co-workers (1995, 1998) further investigated five patients with globoid cell leukodystrophy, who had been treated with allogeneic haematopoietic stem cells. The rationale behind this course of treatment was that donor leukocytes would enter the CNS to correct the enzyme deficiency associated with the disease. These authors noted that a difference between results of transplantation in patients with late-onset, compared to those with onset during early infancy may be due to the different rates of entry of donor-derived mononuclear leukocytes to form perivascular cells or microglial cell populations within the CNS. The course would be slow and would be expected to occur over a period of months to a year after engraftment. In their later study, Krivit and colleagues (1998) demonstrated the reversal of symptoms of enzyme-deficiency in a two-and-a-half years old infant given umbilical cord blood. This particular evidence implied the existence of a microglial progenitor population in human umbilical blood. These experiments lent further support to the concept that resident microglia are long-lived cells, with a very slow turnover, that are able to repopulate their numbers through intrinsic proliferation (Hickey et al. 1992; Perry and Gordon, 1988, 1991). Although these cells are capable of division, this property is arrested once they populate the parenchyma and differentiate into mature process-bearing forms.

A credible explanation for these conclusions would be to consider that the main influx of microglial progenitors takes place within a brief perinatal window in time, whereby microglial progenitors populate the nervous system, and that following the developmental maturation of the blood-brain barrier, such recruitment no longer operates, or is highly restricted. Any subsequent increase in the adult microglial population would be a result of the proliferation of pre-existing parenchymal microglia. An alternative and equally plausible explanation for these findings would be related to a difference in the ontogeny of resident microglia from meningeal macrophages, perivascular macrophages or even perivascular parenchymal microglia. Evidence for this proposal derives from recent emerging evidence on the origin of macrophages that suggests a divergent origin between foetal macrophages during development and bone marrow-derived macrophages of the adult (Morioka et al. 1994; Naito 1993; Naito et al. 1996; Shepard and Zon, 2000; Takahashi 1994; Takahashi et al. 1996). Primitive and foetal macrophages are derived from the yolk sac and liver. The liver in particular, progressively becomes the major site of haematopoiesis until shortly before birth when the bone marrow takes over this task. Hence, for the major part of development, these two principal tissues, the yolk sac and the liver, are predominant sources for primitive macrophages. Foetal macrophages differentiate from primitive macrophages before the development of monocytes and prior to the circulation of blood within vessels. These early macrophages are migratory and distribute throughout the mesenchyme surrounding tissues (including the CNS), and progressively colonise these tissues to be retained as resident macrophages (Takahashi et al. 1996). Later, following the establishment of the blood circulatory network, newly generated monocyte-derived macrophages can supplement the tissue-residing population.

Morphology and distribution of microglia in the developing human nervous system

In the present work we have seen that mononuclear phagocytes of the human foetal cerebrum can derive from two principal sources: (i) from cells located in the mesenchyme and leptomeninges which populate the marginal layer, lower cortical plate and subplate of the telencephalic neocortex during the initial period of the second trimester (12-14GW) and (ii) from vascular-derived progenitors and macrophages which begin to enter via blood vessels within the internal capsule, germinal layers, and at the boundary between deep grey structures such as the caudate and thalamus, around the same period in development. While the first type of progenitors display different morphological and phenotypic characteristics (small round cells, RCA-1$^+$ TL$^+$ CD11b$^-$ CD45$^-$ CD64$^-$ CD68$^-$), and clearly differentiate earlier within the subplate of the human foetal telencephalon, the latter variety (RCA-1$^+$ TL$^+$ CD68$^+$ CD11b$^{+/-}$ CD45$^{+/-}$ CD64$^{+/-}$) has the tendency to adopt amoeboid macrophage-like forms, and associate with neuronal and glial fibers and certain blood vessels and migrates long distances (tangentially and radially) along white matter tracts. Nevertheless, these cells progressively

disperse throughout the nervous system, and can be seen to divide within the intermediate zone of the telencephalon and in the germinal layers, as they differentiate into more ramified cells and downregulate their phenotype. The phases of colonisation are best seen within the neocortex, where the initial phase of migration inwards from the meninges is met during the latter half of the second trimester by an advancing population of foetal microglia migrating outwards from the germinal layers (ventricular and subventricular zones). Within other areas, foetal microglia maintained their prevalence within tracts bordering nuclear grey matter (for example basal ganglia, caudate, thalamus) and rarely penetrated the cortical grey matter. Colonisation of these grey matter areas will likely proceed during the third trimester, and will be the subject of separate ongoing investigations. The marked heterogeneity in phenotype of these foetal microglia likely reflected their state of differentiation and/or specialised functions, regionally within the human foetal brain.

At all stages in human foetal development spanning the second trimester, microglial colonisation was found to associate with blood vessels, vimentin-positive radial glia and later with differentiating GFAP positive astrocytes. Initially, between 12-14GW the infiltration of progenitor cells could be followed from the pial mesenchyme, and these RCA-1 positive cells populated the subplate, lower cortical plate and intermediate zone, where they began to differentiate into ramified cells. A small proportion of these cells, located to the subplate, demonstrated more robust morphologies, more typical with macrophages and expressed CD68. However, by far the majority of the microglial progenitors were negative for monocyte/macrophage markers employed in this study. At later stages (15-16GW onwards), there was clear indication of additional infiltration of newly arrived progenitors within the ventricular zone of the telencephalon, and these appeared to have traversed the walls of blood vessels. Colonisation was in progress at 16GW and proceeded in a co-ordinated manner, and by 22GW, microglia had distributed widely throughout the developing human foetal brain and began to take on the immature forms of resident ramified cells, probably constituting a merger of the two populations of progenitor cells. However, amoeboid microglia still prevailed within the ventricular zone, subventricular zone and corpus callosum and at boundaries between deep grey nuclei (basal ganglia and thalamus) even up to the beginning of the third trimester. The foetal microglia located beneath the cortical plate (within the subplate) were noted to display a more ramified morphology and downregulated phenotype, on account of their advanced stage of differentiation compared to the newly-recruited progenitors arriving via vessels. This was reflected in their respective phenotypes, as CD68 and RCA-1 appeared to label different populations of foetal microglia, emphasised by their apparent uneven distribution at 19GW and 22GW. Although the lectin additionally identified blood vessels, the thickness of sections

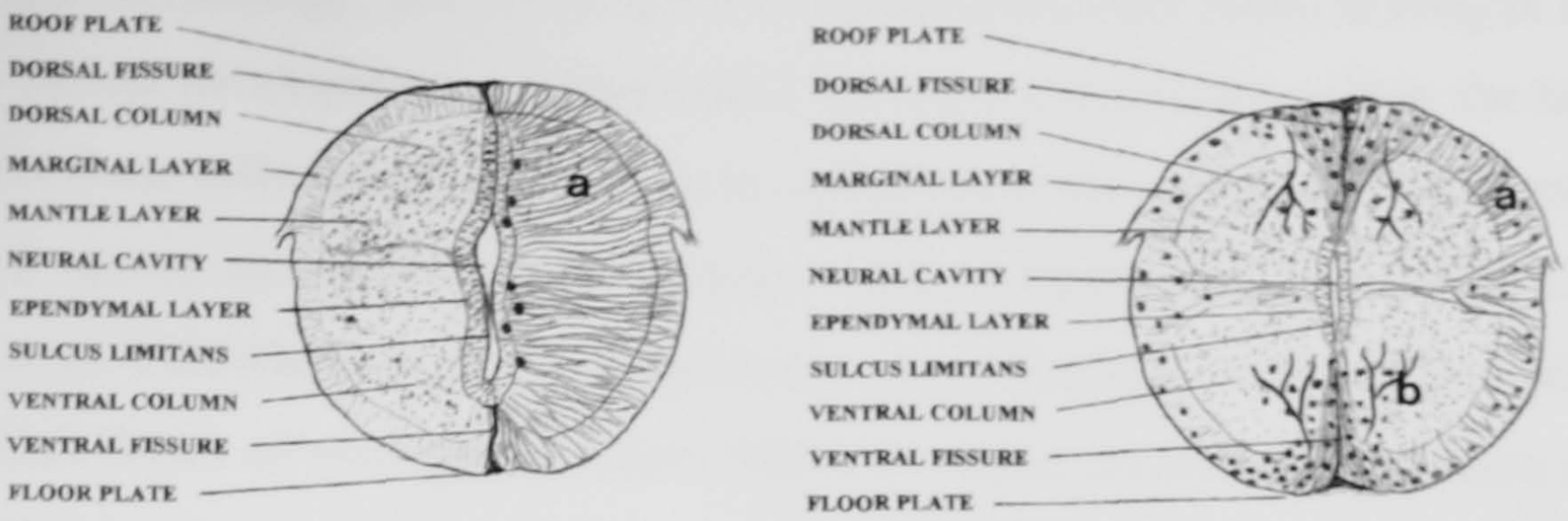
employed in these studies clear enabled morphological discrimination between microglia and blood vessels when viewed under the light microscope.

It has been shown in this study and by others (Gould and Howard, 1987: Larroche, 1982) that the germinal layer is characterised by many thin-walled blood vessels, which lie in the densely compacted cell mass with little support from surrounding tissue, and this is a frequent site of haemorrhage in the preterm infant (Rezaie and Dean, 2002). These vessels are therefore relatively weak although they are at least partially invested by glial elements by the 10^{th} week (Gould and Howard, 1987). In this study, GFAP positive astrocytic investment of vessels within these regions was clearly evident from 19GW, and may indicate the commencement of regional barrier formation between the blood and brain. As the capillaries within the germinal layer offer perhaps the least resistance to migration, this appears to be a logical portal of entry into the nervous system. Microglial progenitors identified within the germinal layer can be seen within or surrounding capillaries and can be identified by microglial markers in this study and by others (Choi, 1981; Peudenier et al. 1991; Wierzba-Bobrowicz et al. 1997; Zecevic et al. 1998). As gestation continues, microglia are increasingly detected at sites distant from the germinal layer, as they disseminate and differentiate throughout the nervous system.

This study has shown that by 22GW, most foetal microglia in the intermediate zone of the telencephalon have differentiated into bipolar, tripolar and multipolar cells, and their immunophenotype was greatly reduced in comparison with the amoeboid forms found within the germinal layers. Microglia maintained their territorial fields and were found to be in a morphologically more advanced state of differentiation in the subplate, beneath the more dense, primitive neurons of the cortical plate. The migration and maturation of microglia, known to be influenced by factors derived from astrocytes, coincided with the development of radial glia and the maturation of astrocytes within the respective brain areas. Postnatally, at six weeks and three months, some RCA-1 positive microglia still prevailed within the corpus callosum, whereas resting adult forms were distributed elsewhere in the cerebrum. Human foetal macrophages were also noted within the optic tract, at the infero-lateral edge of the germinal matrix, around the margins of the anterior commissure and within the white matter of the developing temporal lobe between 16 and 18GW, in accordance with the report by Gould and Howard (1991).

Mononuclear phagocytes in the spinal cord were identifiable by 9GW, at which stage they were predominantly amoeboid in morphology and preferentially located around the neural crest (ependymal zone). These cells expressed CD45 and CD68 and were few in numbers: between 10 and 20 immunolabelled cells could be identified per longitudinal 60μm thick

section of the spinal cord. These cells also co-localised with PECAM-positive blood vessels in the subependymal region, similar to periventricular 'nests' seen in the cerebrum. They were closely associated with GFAP positive radial glia, which were already established at this time. Between 9 and 16GW, microglia appeared as a heterogeneous population, with subsets identifiable using CD11b, CD64, CD68, RCA-1 and less intense expression of CD45. CD68 in particular, consistently identified the majority of amoeboid and ramified microglia within the spinal cord throughout this period. RCA-1 detected cells with more ramified morphologies (2-3 short, fine processes) with advancing gestation. These were morphologically comparable to those detected within the cerebrum. With advancing gestational age, and close to 16GW. there was a marked influx of cells from the meninges/connective tissue (external limiting membrane) into the marginal layer (developing white matter) surrounding the spinal cord. By 16GW GFAP immunoreactivity was largely confined to the marginal layer, associated with microglial influx, which was more prevalent dorsal and ventral to the neural cavity (corresponding to the roof plate and floor plate) with highest densities of microglia (40-50 cells/mm^2). Other regions of the spinal cord (dorso-lateral, medial, ventro-lateral) totalled between 5-30 CD68 immunoreactive cells/mm^2 by 16GW. GFAP immunoreactivity is absent in the neural tube at 7GW, and vimentin is first detected on ventricular cells and radial fibers at 9GW in the spinal cord. Between 12-15GW, GFAP was specifically detected on radial fibers of the anterior and posterior fissures, as well as on short processes within the mantle and marginal zones. The major influx of microglia into the human foetal cord as shown, occurred along the routes of the anterior and posterior fissures and is directed inwards from the mesenchymal tissue/meninges between 14-16GW. As in the telencephalon therefore, colonisation followed the course of radial glia and suggested that cell migration partly follows these tracts (radial migration). In the developing cerebrum, a wave of proliferation and/or migration occurs between weeks 16 and 23, which seemingly supplements the developing cortex with new complement of microglia. As these cells migrate across the intermediate zone, there is evidence for division, since numerous pairs of cells expressing microglial markers and proliferating cell nuclear antigen (PCNA) can be readily identified. This proliferative activity of amoeboid microglia has been documented within the CNS of a number of other species during development (Dalmau et al. 1997; Imamoto and Leblond, 1978; Kaur and Ling, 1991; Kitamura et al. 1984; Ling and Tan, 1974; Ling and Wong, 1993; Marin-Teva et al. 1999; Schnitzer, 1989; Vela-Hernandez et al. 1997). **Figure 99** briefly summarises these findings.



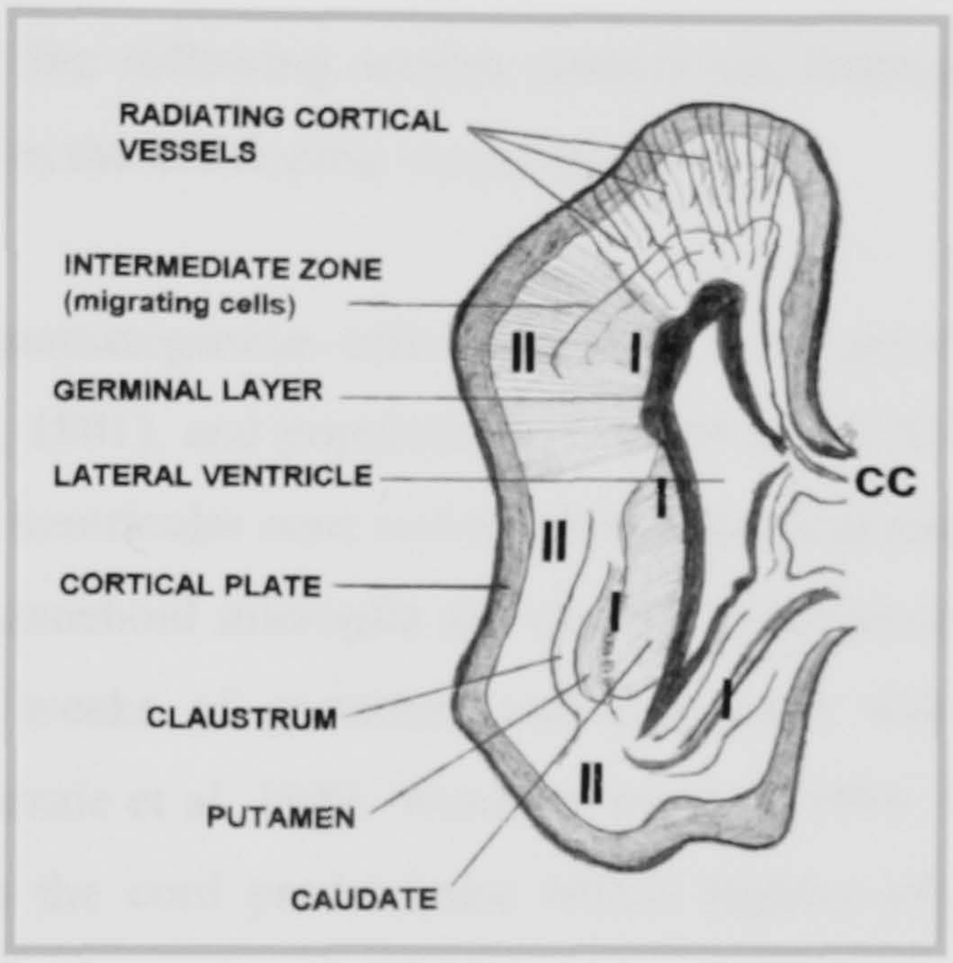


FIGURE 99

Anatomy of the human foetal spinal cord 9-16GW, and cerebrum 22GW

Top: Spinal cord at 9GW. There are a few amoeboid microglia within and overlying the ependymal layer at this stage. However, mononuclear phagocytes are abundant within the meninges/connective tissue surrounding the cord. GFAP positive radial glial fibers (a) course in parallel arrangement from the ependyma to the marginal layer. Spinal cord at 16GW. The dorsal and ventral fissures are major sites of inwardly-directed microglial influx in the spinal cord. Note that radial glial fibers (GFAP-positive) are now redistributed to the marginal layer (a). ICAM-2 positive blood vessels (b) coincide with areas of microglial influx. There are a few transitional forms of microglia within the grey mantle layer. **Lower:** coronal section of one cerebral hemisphere 20-22GW, at the level of the basal ganglia. (I) microglia in the germinal layer, projections of the corpus callosum (CC) and the boundary between the caudate and putamen are amoeboid, whereas those distributed throughout the intermediate zone (II) are transforming early ramified forms in the second trimester. Note the arrangement of radiating cortical vessels, radial glial astrocytes and migrating cells within the intermediate zone.

The work presented here, and in several recent published articles, has helped to paint a clearer picture of the morphology, phenotype and distribution of human foetal microglia in the CNS and retina during development. Morphological accounts of microglia within the human CNS indicate that these cells are already present in limited numbers within the brain between 5 and 8 GW, and appear as round or oval 'globose' cells (Andjelkovic et al. 1998; Choi, 1981; Fujimoto et al. 1989;Geny et al. 1995; Soloveva and Orlovskaia, 1979; Zečević et al. 1998). These earliest forms of microglia (CD68+, MHC II+ cells) occur in low numbers and appear to be inefficient at presenting antigens (Geny et al. 1995). CD68 positive cells have been located in the ventricular and marginal zones between 5 to 7 weeks (Zecevic et al. 1998). Other cells within the nervous tissue are typical of macrophages, and these occur at or near walls of blood vessels. The following section summarises findings related to the regional distribution of microglia in the developing human brain:

***Spinal cord*:** Random haematogenous cells have been noted prior to 10GW in the human foetal spinal cord (Choi, 1981), and populations of CD68 or RCA-1 positive microglia have been detected within the ventricular zone and overlying marginal zone between 5 and 7 weeks (Zecevic et al. 1998). Amoeboid microglia are sparsely distributed within the human foetal spinal cord from 9-10 weeks of gestation, mainly located within the ependymal layer (Hutchins et al. 1992; Rezaie et al. 1999; Weidenheim et al. 1994; Wierzba-Bobrowicz et al. 2001). Microglia within the cord predominate within regions of developing white matter where they coincide with vimentin and GFAP-positive radial glia, and increase in numbers between 10 and 20 GW (Rezaie et al. 1999; Weidenheim et al. 1994; Wierzba-Bobrowicz et al. 2001). Ramified cells with delicate, elongated processes can be detected by 16GW within the grey matter (Weidenheim et al. 1994). Aggregates of microglial progenitors are also situated beneath the pial surface of foetal spinal cords (Rezaie et al. 1999; Weidenheim et al. 1994). Whereas astrocytes and oligodendrocytes appear to differentiate along rostral to caudal gradients within the foetal spinal cord, such a relationship is less clear for differentiating RCA-1 positive microglia (Weidenheim et al. 1994). Furthermore, immunoreactivity for GFAP and myelin basic protein (oligodendrocyte marker) is more intense in anterior and antero-lateral funiculi than in the dorsal and posterolateral funiculi. This suggests a physical temporo-spatial relationship between astrocytes and oligodendrocytes, and these two cell types may be responding to similar growth and differentiation factors (Weidenheim et al. 1994). Alternatively, a mutual influence may exist that drives the correct differentiation of these glial lineages. The same is perhaps true for microglial cells, which coincide within the same localities as macroglia, demonstrated in this study. Intriguingly, synaptophysin reactivity (a synaptic marker) manifests more strongly in the anterior aspects of the spinal cord (Wierzba-Bobrowicz et al. 2001), and could possibly be related to microglial colonisation.

Myelination of the human spinal cord begins from 11 to 16GW (Choi, 1981; Hutchins et al. 1992; Zecevic et al. 1998), and double immunofluorescence labelling has indicated that some of the early haematogenous cells also express myelin basic protein (MBP) before oligodendrocytes have differentiated. The significance of this finding remains to be ascertained.

Cerebellum: Within the cerebellum, amoeboid microglia surround the fourth ventricle and localise in the vicinity of blood vessels within the germinal matrix by 11GW (Wierzba-Bobrowicz et al. 1998, 2000). By 14GW, round, ferritin-immunoreactive microglia are found within the periventricular germinal matrix and at the boundary of the dentate nucleus (Maslinska et al. 1998). These cells progressively proliferate and develop into ramified cells in subcortical areas, reaching densities of ~6 cells/mm^2 by 22GW (Wierzba-Bobrowicz et al. 1998). By 20GW, ramified cells have begun to invade the inner aspect of the cerebellar granular layer, and progressively colonise the outer aspect between 24 and 28GW. Following this period, microglia invade the Purkinje cell layer (primarily in the vermis, subsequently in cerebellar hemispheres) and downregulate their phenotypic markers, as they settle down and adopt mature 'resting' forms (Maslinska et al. 1998). Postnatally, ramified cells can occasionally be detected in the cerebellar white matter.

Mesencephalon: RCA-1 positive cells can be observed in the mesencephalon around 8GW. These mainly occur as isolated amoeboid cells positioned close to the cerebral aqueduct, around the germinal matrix, close to the parenchymal (ablumenal) wall of blood vessels, and in the leptomeninges (Wierzba-Bobrowicz et al. 1995, 1998). A large proportion of lectin-positive cells are preferentially located around the wall of vessels, adherent in rows or clusters. Morphologically, they possess oval or spindle shapes with a foamy cytoplasm full of vesicular material (Wierzba-Bobrowicz et al. 1995). These cells progressively emit short pseudopodal processes at locations associated with the matrix within mesencephalic tectum and tegmentum, and ramify mainly from 16 to 40GW (Wierzba-Bobrowicz et al. 1995, 1997).

Telencephalon and diencephalon: In the frontal, temporal and occipital lobes, ramifying microglia are evident between 11 and 22GW in the expanding intermediate zone (future white matter) (Gould and Howard, 1999; Hutchins et al. 1990; Rezaie and Male, 1997, 1999; Wierzba-Bobrowicz et al. 1998, 2000). These cells are spatially interspersed and occasional microglia may also be detected within the marginal layer (Wierzba-Bobrowicz et al. 1998). Immunoreactivity to MHC class II antigen is confined to cells located in the meninges, choroid plexus, and associated with blood vessels within the periventricular germinal layer and subependymal zone during this period (Esiri et al. 1991; Rezaie et al. 1997; Wierzba-

Bobrowicz et al. 2000). Importantly, mononuclear phagocytes in these locations have been regarded to operate in 'immune surveillance' of the adult CNS (Wekerle et al. 1986). Throughout the second trimester, microglial progenitors (amoeboid migratory cells) are abundant in white matter tracts bordering nucleated structures such as the basal ganglia and thalamus. They subsequently invade these grey matter nuclei at later stages of development (Kershman, 1939; Maslinska et al. 1998; Rezaie et al. 1997). Well-differentiated microglia can be detected throughout the CNS after 35GW (Esiri et al. 1991).

Retina: Colonisation of the retina by microglia also bears mentioning here, since this developmental event corresponds closely with that which takes place within the CNS proper. In the human foetal retina, MHC class I, class II and CD45 immunoreactive microglia are present from 10GW (prevascularisation) and express macrophage S22 antigen from 14GW (postvascularisation). The earlier progenitors enter the retina from the peripheral retinal margin and optic disc, whereas the latter population (which comprise at least 40% of the MHC and CD45-positive cells) appear in association with the retinal vasculature and enter via the optic disc after 14GW (Diaz-Araya et al. 1995). Subpopulations of human foetal microglia in the retina, termed 'paravascular' microglia, accompany the developing vasculature (Diaz-Araya et al. 1995; Penfold et al. 1990; Provis et al. 1996, 1997). Thus two waves of microglial migration also appear to take place within the retina: the first via the ciliary margin prior to retinal vascularisation (these are considered to represent the ramified, non-vessel-associated microglia of the adult retina), and a second wave of macrophage-like MHC class II positive cells that enter via the optic disc, coinciding with the development of retinal vasculature (these cells are thought to become established as vessel-associated para- and perivascular microglia, active in phagocytosing the remnants of eliminated endothelial cells during vascular remodelling (Provis et al. 1996, 1997).

From the above accounts, the main focus for microglial progenitors entering the CNS, has been regarded to occur within and around the germinal layers: ventricular and subventricular zones. Foetal microglia are also distributed within the corpus callosum, thalamus, internal capsule, putamen, caudate, septum pellucidum, anterior commissure, tectum and tegmentum of the mesencephalon, and within the retina throughout the second trimester. A striking similarity in all these investigations is the characteristic 'pattern of differentiation' associated with the progressive outward migration and ramification of microglia from their original portals of entry which, in the telencephalon, has been widely accepted as located within the periventricular germinal layer (see Rezaie and Male, 1999). It has however, emerged from this study that at the earlier time points examined between 12 and 14GW, microglial progenitors are intimately associated with the parenchymal wall of penetrating radial blood vessels, and

appear to invade the CNS parenchyma from the surrounding mesenchyme and align within the subplate region and intermediate zone of the developing telencephalon. At later stages of development (16GW onwards), there is additional supplementary infiltration of progenitors via blood vessels in the ventricular and subventricular zones. This is in keeping with studies carried out in lower vertebrates and mammals (Alliot et al. 1999; Herbomel et al. 2001: Kaur et al. 2001; Sorokin et al. 1992), which indicate that early foetal macrophages first colonise the cephalic mesenchyme from whence they invade the brain, *prior* to commencement of vascularisation of this organ. Subsequently, these cells penetrate the CNS parenchyma in association with the invading vessels. The existence of a dual lineage for microglia (cells derived from foetal macrophages versus bone marrow progenitors) is a topic that is currently under debate (Alliot et al. 1999; Andjelkovic et al. 1998; Kaur et al. 2001; Provis et al. 1996, 1997; Rezaie and Male 1999, 2002; Rezaie et al. 1997), and will be discussed later in this section.

By comparison with normal foetal development, the study of microglia in developmental disorders has been rather neglected. There is some circumstantial evidence from ultrastructural studies that microglia are in a morphologically more activated state (rod-shaped, vesicle-laden) in the CNS of foetuses from mothers diagnosed with schizophrenia (Soloveva and Orlovskaia, 1979). Although not demonstrable in the three cases investigated in the present study, the density of lectin-positive ramified cells in Down's syndrome is reported to be elevated during the second trimester, and microglia appear more prominent when compared to normal foetal CNS (Becher et al. 1991; Wierzba-Bobrowicz et al. 1999). Whether this finding reflects a more activated phenotype of resident microglia (in the sense that they upregulate glycoconjugate residues on their cell surface and thus become more readily detectable) or whether there is an actual increase in cell numbers *per se*, is not clear. Since there is believed to be an inherent disturbance in synaptogenesis in Down's syndrome that takes place essentially beyond the second trimester, it is tempting to speculate that microglia are in some way associated with such events. Whereas no differences have been noted between MHC class II expression by microglia in spinal cords from normal foetuses and those with genetic disorders of Down's syndrome (trisomy 21), Nori's syndrome, mucoviscidosis, or mucopolysaccharidoses, again the numbers of CD68 and RCA-1 lectin positive cells appear to be greater in these disorders (Wierzba-Bobrowicz et al. 2001). Intriguingly, the genes encoding β-subunits for a number of cytokine receptors including IL-3, IL-4, IL-5, IL-9, M-CSF and GM-CSF receptors, are also located in the vicinity of the genetic disturbances affecting mucopolysaccharidoses (Wierzba-Bobrowicz et al. 2001). Whether these are related or merely coincidental remains to be established.

Preferential accumulation and differentiation of microglia within the subplate zone

It is noteworthy that mature forms of microglia are aligned within the subplate at the boundary between the intermediate zone and cortical plate with progressive development. Foetal microglia also form boundaries around the basal ganglia. Presumably these densely cellular sites (grey areas) lack either necessary matrix components or signalling molecules required for colonisation during the early second trimester. Although this distribution pattern can be taken to signify specific stages in the process of colonisation, it is also possible that microglia perform specific roles at these sites, for example in the development of a blood-brain barrier (Mato et al. 1996). It has also been considered that colonisation is a response to programmed neuronal death or axonal degeneration. Despite this, other studies, including the present one, have failed to correlate microglia solely with cell death during foetal development, and this idea is not consistent with the overall distribution of microglial progenitors. Therefore, it appears likely that the process of CNS colonisation by microglia is primarily a developmentally regulated step rather than a response to cell death in a simplistic sense. What therefore, preferentially attracts microglial progenitors to the subplate during the initial period of the second trimester?

In order to address this question, we should first consider the development of this region of the telencephalon in more depth. It is well established that neurons are generated in ventricular zones of the cortical wall and ganglionic eminence and reach their destinations by radial and tangential migration (Meyer et al. 2000). The cortical plate of the human neocortex emerges around the seventh week of gestation, prior to which it differentiates from the preplate and exists as two components, namely Cajal-Retzius (CR) cells in the marginal zone, and subplate cells below the cortical plate. Within the cortical plate, neuronal lamination forms in an 'inside-out' manner (lower layers formed initially and superceded by subsequent layers), and this patterning is governed by proteins released from CR cells (such as reelin or presenilin), and the rudimentary sub-pial granular layer overlying the marginal zone (which harbours tangentially migrating reelin positive neurons) (Meyer et al. 2000; H. Uylings, personal communication). Pioneering neurons within the marginal zone and subplate contribute the first efferent fibers to the internal capsule via the intermediate zone.

The development of the human subplate is a protracted process that extends over several months (Kostovic and Rakic, 1990). Between 13 and 36GW, this transient structure serves as a 'waiting area' for unsettled thalamocortical, corticostriatal and associative and commissural pathways. Furthermore, the subplate acts as a reservoir for subplate neurons that may also exert influence on the laminar arrangement of the cortical plate (Kostović and Judaš, 2002). In particular, plasticity of these subplate neurons appears essential in aiding functional recovery

following perinatal brain injury. Subplate neurons (a proportion of which are nitrinergic), are thought to be involved in the development of connections between the thalamus and the cerebral cortex, specification of cortical areas and formation of ocular dominance columns. These subplate neurons are involved in the early stages of synaptogenesis of the cerebral cortex. Synapses received by these neurons (many of which are transient) are important for transmitting early afferent input to neurons of the cortical plate. A proportion of the subplate neurons will also die, although the timing and mechanisms underlying this have not been confirmed (Kostovic and Judaš, 2002; Rakic and Zecevic, 2000). Furthermore, cells within this region display less vulnerability to hypoxia compared to the intermediate zone and periventricular areas (Kostovic and Judaš, 2002; Rezaie and Dean, 2002), a property that may either reflect the variation of cerebrovascular architecture to these regions, or intrinsic cellular capabilities.

Thalamocortical fibers predominantly accumulate within the subplate between 13 and 24GW, and these are likely to be involved in area specification of the cortex, participating in the induction of a new area. As a result this region is highly plastic. The subplate zone is characterised by large extracellular space that is rich in extracellular matrix components, particularly of the chondroitin sulphate family, laminin, fibronectin, tenascin, as well as growth-promoting molecules, contains early maturing neurons, neurotransmitters and synapses (prior to their detection within the cortical plate). The abundance of extracellular matrix, attractant and repellant components within this area is also central to its role, as changes in the gradients of molecules between the subplate zone and the cortical plate (Hübener et al. 1995; Pearlman and Sheppard, 1996), can promote their relocation from the subplate into the cortical plate (Bicknese et al. 1994; Miller et al. 1995). Hunter and colleagues (1992) found that laminin and s-laminin (the homologues of the B-subunit of laminin, which signals the outgrowth of motor neurons) were transiently present in the subplate of the developing rat, and disappeared with progress in development. Both fibronectin and chondroitin sulphate proteoglycan are prominent in the marginal zone and subplate of the murine CNS between E13 and E14, and immunolabelling of fibronectin is closely associated with radial glial processes, whereas that for chondroitin is more diffuse (Sheppard et al. 1991; Stewart and Pearlman, 1987). As the cortical plate itself matures, the expression of these two ECM components decline (by E18-E19), and that for another component of the ECM, tenascin appears in the marginal zone, subplate and eventually widespread throughout the cortex and subcortical white matter. In man, the rich extracellular matrix environment subsides from the 28th week of gestation (Kostovic and Judaš, 2002).

It is known that macrophages and microglia are capable of synthesising and responding to several proteoglycans including keratan, chondroitin and dermatan sulphate proteoglycans under physiological and pathological conditions (Edwards et al. 1990; Jander et al. 2000; Jones et al. 2002; Jones and Tuszynski, 2002; Moon et al. 2002). Evidence from studies looking at the effects of ECM components on neurite outgrowth, suggests that keratan sulphate and chondroitin sulphate proteoglycans are required to establish boundaries for axonal growth, by potentially inhibiting neurite outgrowth in the developing brain and spinal cord (Jones et al. 2002; Jones and Tuszynski, 2002). By contrast, heparan sulphate proteoglycans may support neuronal outgrowth and promote survival of neurons (Moon et al. 2002). Keratan sulphate is known to be present on microglia, when these have differentiated and show a ramified morphology, but is absent on amoeboid cells and macrophages (Bertolotto et al. 1995; Wilms et al. 1999). Kappler and colleagues (1997) found chondroitin sulphate, expressed in the subplate of the developing neocortex, to enhance neurite outgrowth. Specifically, enzymatic removal of chondroitin sulphate from slices of the brain, has been noted to interfere with neurite adherence and outgrowth in tissue culture preparations (Emerling and Lander, 1996). These experiments strongly suggest that the repellant/anti-adhesive properties of the cortical plate and the attractant/neurite-promoting properties of the subplate and intermediate zone, are reliant on specific and differential expression of bound proteoglycans (Bicknese et al. 1994; Emerling and Lander, 1996). Likewise, cytokine and growth factors that are bound to specific sulphated proteoglycans can promote the migration of mononuclear phagocytes (Hayashi et al. 2001). Factors produced by microglia, such as tissue plasminogen activator may additionally be involved in facilitating neurite outgrowth and pathfinding (Tsirka, 2002), possibly through the processing of ECM components. The proteolytic activity of this system may also establish chemoattractant gradients for microglia. Thus, it would appear that the developing subplate (and intermediate zone) is not only a site which promotes the recruitment of microglial progenitors, but also contributes towards their early differentiation. These hypotheses are further supported in this investigation through (i) the characteristic binding of THP-1 monocytes to the subplate and sub-pial granular layer on sections from the neocortex between 16 and 22GW, (ii) the specific pattern of expression of the chemokine MCP-1 (and to a lesser extent, RANTES), confined to the subplate and lower cortical plate during this period. We shall discuss the latter subject further during the course of this discussion.

Morphologically and phenotypically distinct subpopulations of microglia

Another important finding that is emphasised by this study is the heterogeneity inherent in the phenotype of foetal microglia. The fact that microglia are a heterogeneous population of cells is not, in itself, a new finding (Becher and Antel, 1996; deGroot et al. 1992; Hutchins et al.

1990; Lawson et al. 1990; Ogawa et al. 1993). The heterogeneous phenotype of microglia has conferred difficulty in attempts to distinguish the intrinsic population of microglia from invading monocytes or macrophages in the adult. One proposed discriminatory criteria relates to expression levels of multiple surface markers. For example, rodent microglia constitutively express low levels of CD45 and negligible CD11b, detected by flow cytometry (Sedgwick et al. 1991). These are designated $CD11b^{-}/CD45^{low}$, whereas monocytes and macrophages are typically $CD11b^{+}/CD45^{high}$. Becher and Antel (1996) further demonstrated that immediately *ex vivo* human cultured microglia displayed a $CD11b^{+}/CD45^{low}$ phenotype and undetectable levels of CD14 (Peterson et al. 1995). A more recent attempt at multiple characterisation of phenotype for human microglia has been reported by Dick et al. (1997), whereby freshly isolated resident human microglia could be defined by the following flow cytometric phenotype: $CD45^{low}$:CD4-:CD11b+:$CD11c^{high}$:MHC II+:CD26-:CD14-. Upon activation, microglia adopted the following phenotype: $CD45^{low/medium}$: $CD4^{low}$: CD11b+: $CD11c^{high}$: MHC II $^{+/++}$: CD26-: CD14-. The lack of reliable and specific markers for microglia continues to confound attempts to distinguish between transient populations, incoming cells and resident microglia of the brain.

On the basis of morphology and phenotypic expression, microglia within the normal CNS have been further subdivided into two distinct populations, namely amoeboid and ramified cells. From studies in rodents, it is recognised that rounded or amoeboid macrophages are widely distributed throughout embryonic and neonatal rodent brains, where they predominantly occur close to blood vessels, within the white matter, and become increasingly ramified as they differentiate into microglia (Kaur and Ling, 1991; Perry et al. 1995). Similar findings are also documented in this work, both in human and murine development. Amoeboid cells may represent the precursors of ramified microglia (Imamoto and Leblond, 1978), and during development, a rise in the number of ramified cells parallels a decline in the number of amoeboid cells (Ling and Tan, 1974). Data presented herein, both from work carried out *in situ* in the developing brain, and in tissue culture supports the view that microglia may derive from cells with amoeboid morphology. However, it is not yet clear at this stage, how many of the invading cells actually differentiate into amoeboid microglia *in situ*. Studies to date, have yet to resolve this issue, and are mainly hampered by difficulties in tracking the phenotypically heterogeneous population of cells in development. Certainly, not all amoeboid microglia in the foetus and neonate will survive into adulthood and many undergo apoptosis (Perry 1996), although this is another topic that has come into debate (Wang et al. 2002). Therefore, the exact percentage of microglial progenitors that persist and differentiate to form amoeboid and then ramified microglia are as yet unknown. Nevertheless, estimates have placed this figure somewhere in the region of 30% in rodents.

Although microglia share a broad antigenic profile with blood monocytes, lymphocytes and macrophages (Fujita et al. 1981), data from this study and others have shown that antibodies directed at macrophage-specific antigens, possess differing degrees of specificity for foetal microglia at different stages of development (Hutchins et al. 1990, 1992; Sasaki et al. 1988; Sorokin et al. 1994). Macrophage-specific markers frequently localise to cells in the meninges and choroid plexus, without detecting differentiating parenchymal microglia. This is consistent with the knowledge that many surface antigens are downregulated or 'lost' in the process of 'maturation' of amoeboid cells during development, to the adult ramified form, reappearing when the cells are activated (Hume et al. 1983; Oehmichen et al. 1979; Tsuchihashi et al. 1981; Wood et al. 1979). This is illustrated for human microglia in **Table 14**. **Table 15** summarises the phenotypic markers that have been characterised on human foetal microglia *in situ* or in isolated cell cultures. A schematic illustration of the differentiation and associated phenotpye of microglia during human foetal intrauterine development, is presented in **Figure 100**.

Table 14. Phenotypic markers of human microglia

	Amoeboid	Ramified	Reactive
Immunocytochemistry			
CD4	+	+/-	++
CD11b (Mac-1, C3bi)	++	+/-	++
CD45 (leukocyte common antigen)	++	+/-	++
CD64 (FcγR1)	++	-	++
CD68 (PG-M1, KP-1, EBM-11)	++	-	++
MHC class I antigen	+	+/-	+
MHC class II antigen	+	+/-	++
Lectin Histochemistry			
Ricinus communis agglutinin-1 (RCA-1)	++	+/-	+
Griffonea simplicifolia bandeira (GSB-4)	+	-	+
Enzyme histochemistry			
nonspecific esterase	++	-	++
thiamine pyrophosphatase	+	+	+
Miscellaneous			
keratan sulphate	+/-	+	+/-
acetylated LDL receptor	++	-	++
silver carbonate impregnation	++	++	++

[-] not expressed, [+/-] some expression, [+] weak to moderate expression, [++] significantly upregulated expression

Table 15. Phenotype of human foetal microglia

Antigenic determinant	Reference
CD11b/CD18 (Mac-1, C3bi receptor, $\alpha_M\beta_2$ integrin)	Hassan et al. 1991; Lee et al. 2002
CD11c/CD18 (CR4 receptor, p150.95 antigen, $\alpha_X\beta_2$ integrin)	Pouly et al. 1999
CD36 (class B scavenger receptor)	Coraci et al. 2002;
CD45 (leukocyte common antigen)	Diaz-Araya et al. 1995; Rezaie et al. 1997, 1999, 2002
CD64 (Fcγ receptor type I)	Rezaie et al. 1997, 1999, 2002;
CD68 (PG-M1, KP-1, EBM-11)	Andjelkovic et al. 1998; Esiri et al. 1991; Hutchins et al. 1992; Lee et al. 1992, 2002; Pouly et al. 1999; Rezaie et al. 1997, 1999, 2002; Wierzba-Bobrowicz et al. 1999; Zec evic et al. 1998
CD86 (B7-2)	Lee et al. 2002
HLA DP/DQ/DR (MHC class II)	Esiri et al. 1991; Hassan et al. 1991; Lauro et al. 1995; Lee et al. 2002; Wierzba-Bobrowicz et al. 1997,1999,2000, 2001
HAM-56	Rezaie et al. 1997; Wierzba-Bobrowicz et al. 1998; 1999
L1 (MAC-387)	Gould and Howard, 1991
Ferritin	Maslinska et al. 1998; Wierzba-Bobrowicz et al. 1995, 1998, 1999
α1 antichymotrypsin	Gould and Howard, 1991
nucleoside diphosphatase	Fujimoto et al. 1989
Lectins	
Ricinus communis agglutinin-1 (RCA-1)	Andjelkovic et al. 1998; Hutchins et al. 1990, 1992; Maslinska et al. 1998; Rezaie et al. 1997, 1999; Weidenheim et al. 1994; Wierzba-Bobrowicz et al. 1995, 1997, 1998; Zec evic et al. 1998
Lycopersicon esculentum (tomato lectin)	Andjelkovic et al. 1998; Wierzba-Bobrowicz et al. 1999
Arachis hypogea	Wierzba-Bobrowicz et al. 1999
Triticum vulgaris	Wierzba-Bobrowicz et al. 1999

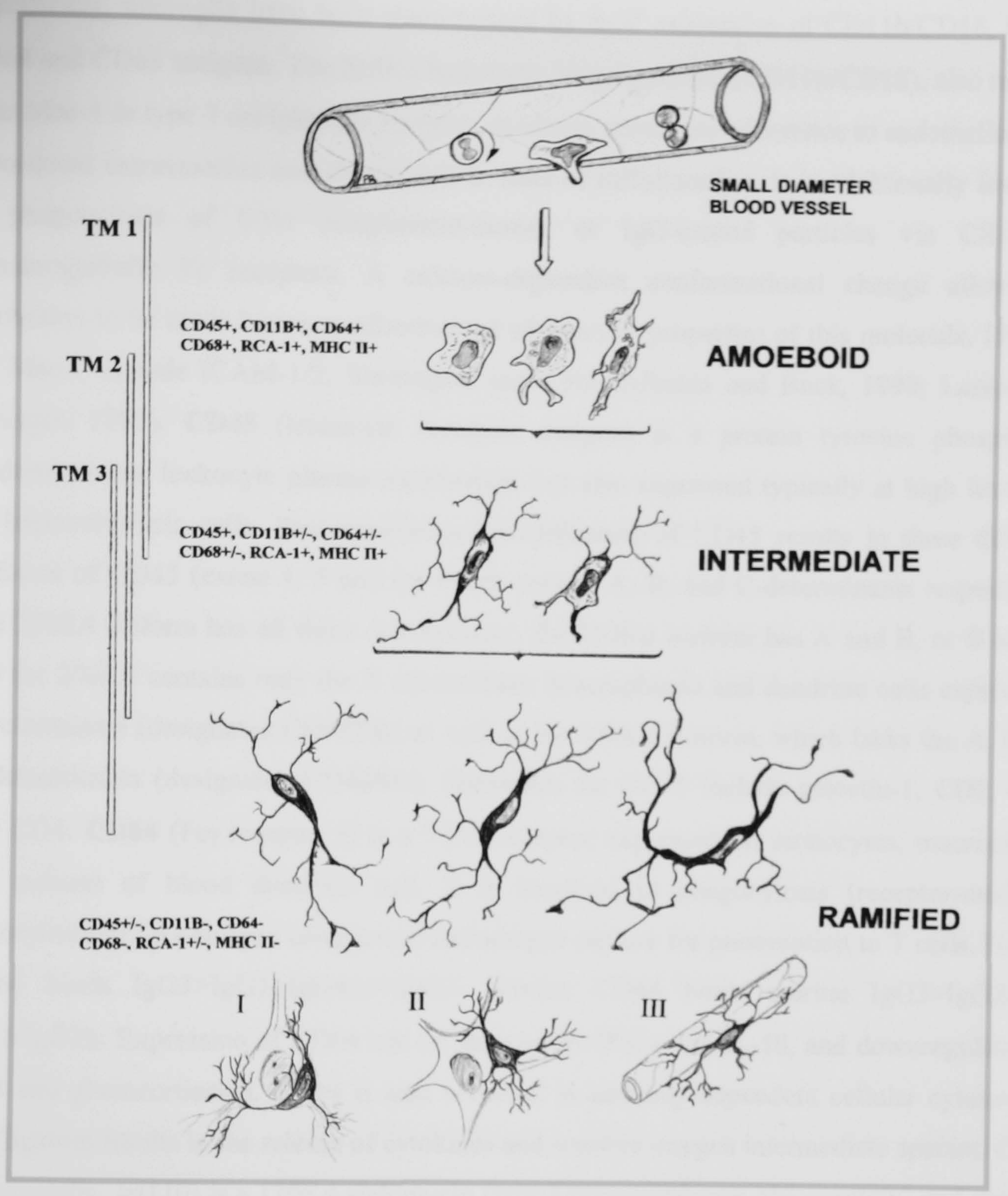


FIGURE 100

The different stages of microglial development

Adapted from original descriptions by Del Rio-Hortega, 1932. The prevailing view has held microglia to be derived from circulating blood progenitors. They have also been considered to be derived from bone marrow precursors, circulating blood monocytes, mesodermal pial elements (meningeal), from intrinsic mesodermal elements, blood vessel adventitia, histiocytes or connective tissue elements. Microglia progressively downregulate surface antigenic markers with maturation. TM1: first trimester, predominantly amoeboid microglia, TM2: second trimester, amoeboid and intermediate stages (transitional, early ramified microglia), TM3: third trimester, predominantly early ramified microglia. In the adult, ramified microglia may be further classified according to their location: (I) neuronal satellites project a number of branches around the nerve cell forming a basket structure, and are more or less flattened against the neuron which they partially surround, or have processes which run in parallel direction to those of the neuron; (II) neuroglial satellites placed near astrocytes or rows of oligodendroglia, and may arborise with branches of the neuroglia; (III) perivascular satellites accompany vessels more frequently in the grey areas, mainly in superficial regions and along larger vessels up to the point where they branch. Capillaries have microglia arranged along their course. In most cases, microglia in contact with vessels are multipolar elements that either run across the vessel walls or are parallel to the latter, in which case their wavy projections extend along the vessels. Descriptions modified after Del Rio-Hortega, 1919 and 1932.

Specifically, microglia have been characterised by their expression of CD11b/CD18, CD45, CD64 and CD68 antigens. The 265Kd leukocyte integrin αMβ2 (**CD11b/CD18**), also referred to as Mac-1 or type 3 complement receptor, mediates monocyte adherence to endothelium and subsequent extravasation and chemotaxis to sites of inflammation. It is additionally involved in phagocytosis of C3bi complement-coated or IgG-coated particles via CR1 and immunoglobulin Fc receptors. A calcium-dependent conformational change allows the distinction to be made between adhesive and phagocytic properties of this molecule. Ligands for Mac-1 include ICAM-1/2, fibrinogen, and C3bi (Albelda and Buck, 1990; Larson and Springer, 1990). **CD45** (leukocyte common antigen) is a protein tyrosine phosphatase predominant on leukocyte plasma membranes, but also expressed typically at high levels on all haematopoietic cells. Post-translational modification of CD45 results in three different isoforms of CD45 (exons 4, 5 and 6), which encode A, B, and C-determinants respectively. The 220Kd isoform has all three determinants, the 210Kd isoform has A and B, or B and C, and the 200Kd contains only the B determinant. Macrophages and dendritic cells express the B determinant (designated CD45RB) as well as the 180Kd isoform, which lacks the A, B and C determinants (designated CD45RO). Substrates for CD45 include galectin-1, CD2, CD3, and CD4. **CD64** (Fcγ receptor I) is a 72Kd receptor expressed on monocytes, macrophages and subsets of blood dendritic cell. It is involved in phagocytosis (receptor-mediated endocytosis of IgG-antigen complexes) and antigen capture for presentation to T cells. Human CD64 binds IgG3>IgG1>IgG4>>>IgG2. Murine CD64 binds murine IgG3>IgG2a>>> IgG1/IgG2b. Expression of CD64 can be induced by IFN-γ and IL-10, and downregulated by IL-4 and glucocorticoids. CD64 is also involved in antibody-dependent cellular cytotoxicity and ligation results in the release of cytokines and reactive oxygen intermediate species. **CD68** (macrosialin, gp110) is a 110Kd sialomucin (type I transmembrane glycoprotein) and member of the host defence scavenger receptor superfamily (SR-D class). It is expressed intracellularly in cytoplasmic granules and on the surface of monocytes, macrophages, dermal dendritic cells, Langerhans cells, myeloid progenitor cells and a subset of CD34+ haematopoietic bone marrow progenitor cells. In mice, this receptor has been shown to bind to oxidatively-modified low density lipoprotein (LDL).

A battery of these macrophage-specific markers were employed in the present investigation and have clearly shown the variability in antigenic profile of subpopulations of microglia in the human foetal brain during the second trimester. Within the ventricular and subventricular zones and the corpus callosum, foetal microglia expressed higher levels of all these markers, and assumed morphologies more characteristic of phagocytic cells within these regions. In disseminating from these sites, foetal macrophages downregulated their expression of CD64 and CD68. Morphologically, these isolated cells (mainly encountered within the intermediate

zone and subplate) adopted bipolar and multipolar forms. Lectin histochemistry identified a similar progressive pattern of downregulated phenotype. Surprisingly, a minor population of CD68 positive cells also expressed the selectin CD62-P on their cell body, which has not been reported in the developing CNS. These cells were randomly distributed throughout the foetal cortex and not usually placed close to vessels. Most were round or angular in morphology, and a few appeared to be phagocytic, displaying similar morphology to amoeboid microglia. Although the expression of CD62-P has not been reported on microglia, a previous study showed CD62-P mRNA to be induced in Kupffer cells of the liver, following activation (Essani et al. 1995).

What determines the phenotypic heterogeneity of microglia?

Put simply, the phenotype of microglia depends on their location, state of differentiation and potential physiological functions. The altered immunophenotype of microglia in the brain appears to correlate with cells in particular localities, and may reflect functional specialisation of microglia in response to local environmental cues. For example, cells which are located in transitory white matter (intermediate zone), and which co-localise with radial glial astrocytes as shown here and in the study by Hutchins et al. (1990), appear to downregulate expression of CD68 and CD64 (phagocyte markers) and become more ramified. Radial glia are demonstrable by the 8th week of gestation in the spinal cord and by the 10th week in the cerebrum (Choi, 1981; Rakic, 1981). Penfold and co-workers (1991) have also reported subpopulations of CD68 and CD45 positive microglia in the human foetal retina. The substrate to which microglia adhere has an important influence on their phenotype both *in vitro* (Giulian et al. 1995), and *in situ*, as discussed previously with reference to the subplate. By comparison, phenotypic heterogeneity is a phenomenon that is seen in other populations of mononuclear phagocyte within foetal tissues, that also reflects the localisation and associated specialised function of these cells. Examples are the spleen (Takeya and Takahashi, 1992; Wijffels et al. 1994), foetal rat lungs (Higashi et al. 1992), and foetal liver (Bardadin et al. 1995; Naito et al. 1990). In these tissues, there is similar uncertainty whether heterogeneity reflects variation within a single cell population, or identifies the coexistence of several distinct populations of macrophages.

The foetal brain represents a distinctive environment for the differentiation of mononuclear phagocytes. Astrocytes may be partly responsible in controlling this process through cell/cell interactions and via cytokines, particularly M-CSF and GM-CSF, which can modulate cell morphology, function and metabolism (Naito et al. 1996). Studies in M-CSF deficient mice however, have shown only partial loss of microglia (which are reduced in numbers by approximately 30-40%) without significant alterations in their distribution (Blevins and

Fedoroff 1995), despite severe depletion of mononuclear phagocytes in liver. spleen and thymus (Wiktor-Jedrzejczak et al. 1991). These studies have shown that the development of mononuclear phagocytes which is considered to depend on M-CSF during foetal and neonatal life, actually varies from tissue to tissue (Blevins and Fedoroff, 1995; Cecchini et al. 1994; Wijffels et al. 1994). In the brain, the production of GM-CSF. IL-3 and IL-6 by astrocytes, endothelium or even neurons, may partly compensate for the lack of M-CSF (Blevins and Fedoroff, 1995).

The blood-brain barrier (BBB) is also known to influence microglial phenotype (Becher and Antel, 1996). In the adult CNS, microglia express higher levels of phenotypic markers at sites lacking a BBB, and this also occurs in aged CNS where presumably limited barrier breakdown occurs, allowing leakage of plasma proteins. A full barrier forms relatively late in development, long after the majority of neurons have formed their connections (Davis et al. 1994). However, cerebral endothelial cells have an intrinsic ability to form 'tight junctions' when they first invade the brain during development (Saunders 1992), and naturally-occurring plasma proteins have not been found to leak across even the most immature cerebral vessels, although the 'BBB' of the foetus and newborn is much more permeable to smaller molecules. Hence microglia in different locations will be in contact with different levels of serum molecules, which is partly related to their distance from the blood vessels and the ependymal barrier. Moreover, different areas of the brain may develop their barrier properties at different times. In the mouse, a 'mature barrier' is lacking prior to embryonic day 15 (Engelhardt and Risau, 1995). A barrier first forms in the spinal cord and finally in the telencephlon. There appears to be an ependymal-cortical gradient in barrier formation, since injected proteins are still visible in subependymal layers at embryonic day 16 (Engelhardt and Risau, 1995). The morphology and immunophenotype of foetal microglia may therefore relate partly to the timing and extent of barrier development in each region of the brain.

The mechanisms of entry and dispersion of microglial progenitors in the developing CNS

While this work has painted an unambiguous picture of the timing and phases of colonisation for microglia in the developing CNS, the mechanisms governing entry. dissemination and differentiation of microglial progenitors are only partially clear. Astrocytes appear to be important and partly responsible for these events, and some potential signals have been discussed extensively elsewhere (Rezaie and Male, 2002), outlined in **Figure 101**. Briefly, factors that have been proposed to drive colonisation of these cells, include the growth factors M-CSF/GM-CSF, chemokines, the onset of synaptogenesis, programmed cell death and specific developmental expression of adhesion molecules and ECM components such as laminin.

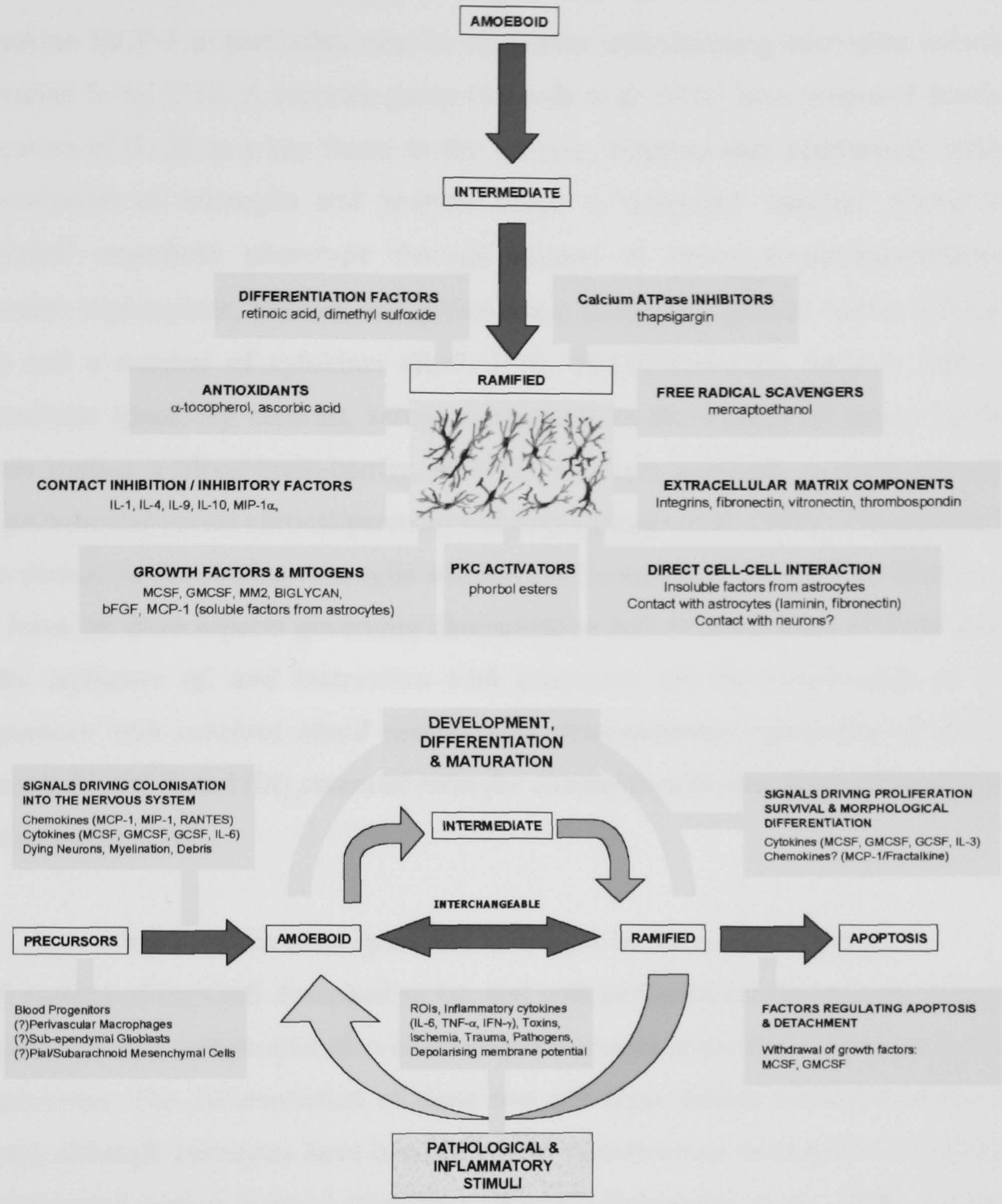


FIGURE 101

Factors associated with the ramification, differentiation and apoptosis of microglia

Data accrued from this investigation suggest that the adhesion molecule ICAM-2 and chemokine MCP-1 in particular, may be associated with directing microglial colonisation in the human foetal CNS. A separate group (Schwab et al. 2001) have proposed developmental expression of IL-16 as a key factor in this process. Neurons may additionally influence the differentiation of microglia and promote either a 'quiescent' ramified phenotype or an 'activated' amoeboid phenotype through release of neurotransmitters/neuromodulators, adenosine triphosphate, purines and pyrimidines, hormones, growth factors ($TGF\beta_2$, NGF, NTs) and a number of cytokines (IL-3, IL-6, M-CSF/GM-CSF, MCP-1, MIF) into the extracellular space. By contrast, serum components in the vicinity of newly formed blood vessels lacking a blood-brain-barrier, probably maintain microglia in more activated states (i.e. perivascular versus cortical/parenchymal cells (Tanaka et al. 1998)). The precise nature of these stimuli on developing microglia will have to be explored further. The following section will focus on three aspects governing dissemination and differentiation of these progenitors: (i) ***the influence of, and interaction with astrocytes***, (ii) ***the relationship of microglial progenitors with cerebral blood vessels, and developmental expression of the adhesion molecule ICAM-2***, and (iii) ***potential roles for chemokines in directing cell migration within the developing CNS***.

The relationship between astrocytes and microglia in the second trimester

In all regions of the CNS discussed so far, and with particular reference to the telencephalon, the differentiation and proliferation of microglia increases in parallel with a rise in the number of astrocytes. The differentiation of these two cell types further coincides within the same regions, although astrocytes have been estimated to outnumber microglia 4-5 fold during this developmental period (second trimester) (Wierzba-Bobrowicz et al. 1998). In the present study, we have seen that microglia co-localise with differentiating astrocytes in the germinal layers (VZ and SVZ), corpus callosum, cavum septum pellucidum and within neural tracts, particularly surrounding deep grey structures such as the striatum and thalamus. Furthermore, they appear to disperse throughout the intermediate zone and adopt more mature morphologies, *prior to* and *concomitant with* the differentiation of GFAP-positive astrocytes within this region, which takes place around 19-23GW. Curiously astrocytic differentiation within the intermediate zone follows a similar spatially interspersed pattern as seen for foetal microglia. Immediately before and during this period, foetal microglia can be seen to specifically associate with vimentin-immunoreactive radial glial fibers that span the neocortex and are particularly rich within the subplate. A similar pattern of events appears to take place within the spinal cord between 9 and 16GW. These observations suggest that the differentiation of microglia precedes that of astrocytes, although it remains to be seen whether factors released by microglia may influence astrocyte differentiation *in situ*. This is

particularly intriguing, since an overwhelming number of studies have focussed on the contrary relationship, that is to say, the influence of astrocytes on microglial differentiation.

How do these findings relate to our present knowledge of the differentiation of astrocytes *in situ* during development? The intermediate filament protein vimentin, which is developmentally expressed in proliferating neuroepithelial cells, is among the first markers for immature and differentiating astrocytes and their progenitors. GFAP on the other hand has long been recognised as specifically restricted in its expression to astrocytes that have gone (or are undergoing) differentiation to mature forms, and subsequently lose expression of vimentin (Bignami and Dahl, 1974; Bignami et al. 1980; Eng et al. 1971: Wilkinson et al. 1990). The appearance of GFAP positive cells has been reported to correlate with myelination in rodents (Bignami et al. 1980 Jacque et al. 1980), and in humans (Takashima and Becker, 1983). However, the timing of proliferation and differentiation of astrocytes, which occurs from the second trimester onwards, is at odds with this hypothesis, since myelination occurs mainly within the third trimester and after birth (Reske-Nielsen et al. 1987b). Notwithstanding, the relationship between differentiating astrocytes, microglia and maturing oligodendrocytes is one that will require further analysis during this period.

Expression of GFAP has been identified on human foetal radial glia as early as 9GW (Wilkinson et al. 1990) and on more mature, process-bearing foetal astrocytes between 14-15GW (Roessmann and Gambetti, 1986a; Wilkinson et al. 1990), although at this age, there is very little expression (Aquino et al. 1996). Aquino and colleagues (1996), like Borit and McIntosh (1981) before them, observed GFAP positive tanycytes and many small immunoreactive cells in the ventricular and subventricular zones at 16GW. Throughout the second trimester, bipolar cells and radial glia are evident in the cerebral hemispheres (Borit and McIntosh, 1981). In their study, Roessmann and Gambetti (1986a) noted GFAP positive astrocytes to form aggregates at the borders between large white matter bundles/fiber tracts and neighbouring grey areas, lining the cavum septum pellucidum, and over the surfaces of the fornices and optic tract. These initial findings have been verified convincingly in the present work. Astrocytes have been found in many developing fiber tracts including the internal capsule, cortico-spinal and medullary tracts, where they begin to differentiate from 19-20GW onwards (Roessmann and Gambetti, 1986a; Wilkinson et al. 1990). Differentiated astrocytes populate the CNS in a systematic way and are more widespread and readily discerned by the end of the second trimester (Reske-Nielsen et al. 1987a; Wilkinson et al. 1990). By 22GW, GFAP positive cells have been noted by Aquino and colleagues (1996) within the marginal layer, a few cells identified in the subplate region, and continuing expression widely distributed in paraventricular areas. These authors also found a striking

increase in the content of GFAP within the prefrontal cortex from 16-24GW, and the rapid increase towards the late second trimester was thought to correlate with the active proliferation of GFAP positive cells. The overall increase in levels of GFAP was along a caudal-rostral gradient, consistent with the maturation of the CNS during the foetal period. Therefore the findings presented in this work correspond well with previous reports, and indicate that the differentiation (and dissemination) of astrocytes parallels that for microglia during the second trimester.

Three additional and separate points also surfaced from this study. These relate to (i) the timing of ensheathment of cerebral blood vessels by astrocytic endfeet, as a precursor to establishment of blood-brain barrier properties, (ii) expression of GFAP by specialised cells, termed tanycytes, that line the ventricles, and (iii) the pathology of foetal astrocytes in response to hypoxic-ischaemic injury. With regards to the first point, a clear finding from the present study was the region-specific investment of radial cortical blood vessels and germinal layer vessels, with endfeet derived from differentiating astrocytes, detected from 19GW onwards. There appeared to be a gradient for ensheathment of cortical vessels, with those in more dorsal and medial aspects of the telencephalon preferentially targeted. This may bear some significance with respect to the development of properties associated with the blood-brain barrier, that are region-specific (i.e. some areas develop these properties in advance to others). Partial ensheathment of blood vessels by astrocytes has been detected by electron microscopy from 10GW (Povlishock et al. 1977). GFAP positive endfeet (considered to derive from type I astrocytes: Miller et al. 1985), have also been noted around vessels from 14GW in the brainstem and cerebrum, and from 17GW in the ganglionic eminence (Wilkinson et al. 1990). Studies *in vitro* indicate that type II astrocytes are prevalent prior to the second trimester, whereas type I astrocytes predominate around 16GW. From these, one can speculate that the differentiating GFAP positive astrocytes that are detected around these vessels correspond to type I astrocytes, and certainly *in vitro* studies, have shown these cell types to influence microglial morphology, phenotype and functional properties. Thus type I astrocytes may potentially emerge *in situ*, within the human cerebrum towards the end of the second trimester and further influence microglial activity and differentiation beyond this period.

The second point very briefly addresses tanycytes, which are specialised epithelial cells that may also give rise to astrocytes. Expression of GFAP by tanycytes has been reported previously (Aquino et al. 1996; Gould and Howard, 1987; Gould et al. 1990; Roessmann et al. 1980), and is known to diminish at later gestational stages (Sarnat, 1992). Their density has been detailed to be highest around the third ventricle and adjacent areas of the lateral ventricles. These findings correspond closely with that seen in the present study, where

tanycytes expressing GFAP were not continuous around the ventricular lining, but found regionally, and more frequently around the ventral aspects of the ventricles. This may suggest differential patterns for the differentiation of these cells to astrocytes, or relate perhaps to functional specialisation of these cells. In accordance with the previous studies, the numbers of GFAP-expressing tanycytes were found to reduce considerably, towards the end of the second trimester.

The third point concerns the rather remarkable pathological forms of astrocytes that were prevalent in the foetal case with hypoxic-ischaemic injury. It has been noted that a variety of cases with perinatal brain injury (ranging from subependymal haemorrhage, ponto-subicular necrosis, through to periventricular leukomalacia), show typical pathological alterations within the white matter, and specifically the periventricular white matter (Rezaie and Dean, 2002a, 2002b). Immature GFAP positive astrocytes are capable of a reactive response from as early as 20GW. Changes such as axonal injury or damage to oligodendrocyte precursors, that may be the result of hypoxic-ischaemic injury, or bacterial infection, are often accompanied by such glial activation (Roessmann and Gambetti, 1986b). The morphology of reactive 'gemistocytic' astrocytes in the case with hypoxic-ischaemic injury presented here was comparable to that previously described in perinatal brain injury (Roessman and Gambetti, 1986a), in methylmercury poisoning *in utero* (Choi et al. 1978), in tuberous sclerosis (Stefansson and Wollmann, 1980), cerebellar neuroecto-mesenchymoma (Akimoto et al. 1994), as well as in hypoxic-ischaemic brain lesions (Nakamura et al. 1986). These cells have been considered to react in response to pathological changes particularly involving hypoxic-ischaemic white matter injury (Brand and Bignami, 1969; Gilles and Murphy, 1969; Nakamura et al. 1986; Simpson et al. 1987). Specifically, the response shown in the case with hypoxic-ischaemic injury was diffuse and widespread within white as well as grey areas, particularly affecting cells in perivascular positions, which is within reason given the nature of the pathological insult.

Gemistocytic astrocytes have been described more extensively in studies on human gliomas, where they are considered to represent a 'benign' secondary, and 'biologically harmless' component (Hoshino et al. 1975; Liberski, 1998; van der Meulen et al. 1978). These cells, which typically show marked hypertrophy, rounded morphology, with circular arrangement of bundles of glial filaments, are mainly detected from mid-intrauterine development onwards. They have also been described to accompany abnormal neuronal migration and deranged cerebral cortical organisation following foetal methyl mercury intoxication (Choi et al. 1978). The presence of large numbers of these types of astrocytes therefore indicates some form of ongoing destructive process that most likely interferes with correct neuronal migration and

positioning, and can be indicative of a long-standing reaction. However, it is intriguing that these gemistocytic astrocytes are not usually associated with reactive microglia or macrophages in these disorders. Furthermore, there is debate as to whether in this immature reactive state, these cells are capable of some non-specific phagocytic activity. Focal accumulation of gemistocytic astrocytes (for example at subependymal locations) may alternatively suggest a disturbance in their function that incapacitates their own migration. A separate hypothesis that needs to be addressed is whether these gemistocytic cells reflect an abnormal differentiation of glial (oligodendrocyte and astrocyte) progenitors, which may be arrested at a stage prior to differentiating into mature astrocytes. Gemistocytic astrocytes frequently have a dense eosinophilic cytoplasm, with the nucleus located at the periphery, and are densely filled with a mixture of vimentin and GFAP immunoreactive fibers, and other components (Akimoto et al. 1994; Liberski, 1998). These attest to an immature or abnormally differentiated state. In this context, one should also consider the so-called 'bouquet' cells, which express GFAP in the normal foetal brain, but resemble gemistocytic astrocytes to some extent, in their immature morphology (Borit and McIntosh, 1981). This particular observation may be significant, since these 'bouquet' cells are especially numerous in areas of neural tracts, immediately prior to myelination, and decrease in numbers once myelination is established (Borit and McIntosh, 1981).

The interaction between astrocytes and microglia in tissue culture

Interactions between human foetal microglia and astrocytes were clearly evident from the studies carried out in tissue culture preparations, presented in Chapter IV. Following isolation, human foetal microglia adopted an amoeboid morphology that transformed into morphologically heterogeneous microglia bearing processes when co-cultured with astrocytes. Microglia maintained in these co-cultures were noted to alter their morphology continuously with constant extension and retraction of cellular processes. Migration of these cells appeared random overall, but was confined to a localised 'territory' that was actively scavenged and cleared of debris. However, both the morphology and motility of these cells was reliant on the state of activity and confluency of astrocytes *in vitro*: microglia were highly motile in subconfluent cultures, where astrocytes were differentiating and proliferating markedly, and progressively more stable, and ramified cells when astrocytes had differentiated and attained confluency. Using time-lapse video imaging, human foetal microglia were found to be capable of migration not only in the amoeboid, but also in the ramified state overlying confluent astrocytes, albeit more slowly. Thus, the behaviour of microglia appeared to be governed to a considerable extent by the state of differentiation of astrocytes *in vitro*. Frequently, one could also detect some direct interaction between solitary microglial cells and differentiating astrocytes in these preparations, which may reflect that which occurs *in situ*.

Direct interactions between astrocytes and microglia may of course, be mediated via cytokines, and microglia may influence the proliferation of astrocytes through release of cytokines such as IL-1 (**Table 16**). However, cultured astrocytes have also been shown to respond to microglia-conditioned medium by upregulating ECM molecules such as tenascin on their surface by as much as two-and-a-half fold. The synergistic effect of TGF-β released from microglia and bFGF from astrocytes are believed to account for this upregulation (Smith and Hale, 1997). TGF-β itself inhibits astrocytic proliferation, and can suppress the mitotic effects of EGF and FGF on astrocytes (Lindholm et al. 1992; Rozovsky et al. 1998), and downregulates ICAM-1 expression on astrocytes (Xiao et al. 1996). Microglia themselves have been shown to express receptors for all three isoforms of TGF-β, but it is unclear whether they respond to this cytokine by proliferation (Dobbertin et al. 1997), or whether proliferation is inhibited (Jones et al. 1998). In the former study, the effect of TGF-β was synergistic with M-CSF produced by astrocytes. Therefore it is plain that factors released from microglia can influence the capacity of astrocytes to proliferate as well as regulate their expression of adhesion molecules and ECM components. These effects are of course mutual, and numerous studies have defined the proliferative influence of astrocytes on microglia maintained in culture.

The differentiation of mononuclear phagocytes outside the CNS is known to be driven by sequential actions of different cytokines, such as stem cell factor (SCF), IL-3, M-CSF and GM-CSF (Nicola, 1989). Different studies have addressed the question whether these cytokines are present in the developing brain, and whether microglia respond to them in culture. Genes for a number of cytokines (SCF, IL-3, IL-7, IL-9, CSFs) have been detected early in the developing nervous system (Merrill and Jonakait, 1995). SCF is present in discrete cell clusters in the floor plate of the neural tube, in the early embryonic thalamus and olfactory epithelium. Studies on rodent brains demonstrated that IL-7, IL-9 and M-CSF are expressed as early as E13, and receptors for these factors are co-expressed in the same brain regions, and sometimes on the same cell. Various studies indicate that M-CSF, GM-CSF, IL-3 (produced by foetal astrocytes and microglia) (Blevins and Fedoroff, 1995; Giulian and Ingemann 1988; Lee et al. 1992, 1994; Liu et al. 1994; Malipiero et al. 1990; Mizuno et al. 1994; Raivich et al. 1994; Sawada et al. 1990; Schlichter et al. 1996; Tanaka and Maeda, 1996) or adenosine (Gebicke-Haerter et al. 1996) may act as possible signals for microglial division, whereas deprivation may lead to apoptosis. More specifically, *in vitro* studies indicate that either GM-CSF (Lee et al. 1994) or GM-CSF like peptides in rodents (Giulian et al. 1991), are responsible for microglial proliferation during embryogenesis. GM-CSF has further been shown to inhibit MHC class II expression on microglia, and may keep these cells in an immunologically quiescent state, favouring their differentiation (Hayashi et al. 1993).

Table 16. Functional biochemistry of human foetal microglia

Biological Component	
Cytokines and growth factors	
Interleukin-1 (IL-1, IL-1β)	Hu et al. 1999; Lee et al. 1993, 1994, 2002; Sebire et al. 1993
Interleukin-6 (IL-6)	Chao et al. 1994; Lafortune et al. 1996; Lee et al. 1993, 1994, 2002; Sebire et al. 1993; Sheng et al. 1995
Interleukin-8 (IL-8)	Ehrlich et al. 1998; Janabi et al. 1999; Lee et al. 2002; Lipovsky et al. 1998
Interleukin-10 (IL-10)	Lee et al. 2002; Sheng et al. 1995
Interleukin-12 (IL-12)	Lee et al. 2002
Interleukin-15 (IL-15)	Lee et al. 1996, 2002
Interleukin-16 (IL-16)	Schwab et al. 2001
Tumour necrosis factor-alpha (TNF-α)	Chao et al. 1995; Deguchi et al. 1996; Fine et al. 1999; Lafortune et al. 1996; Lee et al. 1993, 2002; Liu et al. 1996; Sheng et al. 1995
Chemokines	
Macrophage inflammatory protein-1α (MIP-1α)	Hua and Lee, 2000; Lee et al. 2002; Janabi et al. 1999; McManus et al. 1998, 2000; Peterson et al. 2000; Rezaie et al. 2002; Si et al. 1996
Macrophage inflammatory protein-1β (MIP-1β)	DiPucchio et al. 1996; Hua and Lee, 2000; Janabi et al. 1999; Lee et al. 2002; McManus et al. 1998; 2000; Peterson et al. 1997; Si et al. 1996
Macrophage chemotactic protein-1 (MCP-1)	Hua and Lee, 2002; McManus et al. 1998, 2000; Peterson et al. 1997;
RANTES*	DiPucchio et al. 1996; Hu et al. 1999; Hua and Lee 2000; McManus et al. 2000; Si et al. 1996; Hua and Lee, 2000
Interferon-gamma inducible protein-10 (IP-10)	Janabi et al. 1999
Growth-related oncogene alpha (GRO-α)	Hua and Lee 2000
I-309	
Growth factors	
basic fibrobalst growth factor (bFGF)	DiPucchio et al. 1996; Presta et al. 1995
macrophage colony-stimulating factor (M-CSF/CSF-1)	Lee et al. 1993
granulocyte-monocyte colony-stimulating factor (GM-CSF)	Lee et al. 1994
platelet avtivating factor (PAF)	Jaranowska et al. 1995
Potential cytotoxic factors	
nitric oxide	Colasanti et al. 1995; Ding et al. 1997; Peterson et al. 1994
quinolinic acid	Espey et al. 1997
Enzymes	
inducible nitric oxide synthase (iNOS)	Colasanti et al. 1995; Ding et al. 1997
matrix metalloproteinases 1,2,3,9	Cross and Woodroofe 1999; Ghorpade et al. 2001
nicotinamide adenine dinucleotide phosphate (NADPH) oxidase complex components (e.g. gp91-phox)	Green et al. 2001
Receptors	
IL-1RI, IL-1RII, IL-5R, IL-6R, IL-8, IL-10R, IL-12R, IL-13R, IL-15R	Lee et al. 2002
FGF receptors(1-4)	Balaci et al. 1994; Presta et al. 1995
Complement 5a receptor (C5aR, CD88)	Gasque et al. 1997
CD14 (LPS receptor)	Peterson et al. 1995
CD36 (class B scavenger receptor)	Coraci et al. 2002
M-CSF receptor	Liu et al. 1994
GM-CSF receptor	Jaranowska et al. 1995
κ-opioid receptor	Chao et al. 1996
μ-opioid receptor	Chao et al. 1997; Lipovsky et al. 1998; Peterson et al. 1995
orphan opioid receptor (OR)	Chao et al. 1998
substance P receptor (neurokinin-1)	Lai et al. 2000
α-chemokine receptor: CXCR4	Gabuzda et al. 1998; He et al. 1997; Rezaie and Male, 1999; Rezaie et al. 2002; Vallat et al. 1998
β-chemokine receptors: CCR2, CCR3, CCR5	Cross and Woodroofe, 1999; Gabuzda et al. 1998; He et al. 1997; Hegg et al. 2000; Kitai et al. 2000; Rezaie and Male, 1999; Rezaie et al. 2002; Vallat et al. 1998
Ion Channels	
inward K+ channel, outward calcium-dependent K+ channel transiently activated anion channel	McLarnon et al. 1997
Miscellaneous	
substance P	Lai et al. 2000, 2002
NG2 chondroitin sulphate	Pouly et al. 1999

* Abbreviation for: 'regulated on activation, normal T cell-expressed and secreted'

M-CSF appears to affect both the survival and differentiation of microglia. Tomozawa and co-workers (1996) found that 1ng/ml of recombinant human M-CSF enhanced the survival rate of rodent microglia in culture. They also found that 5ng/ml rhM-CSF or 1ng/ml recombinant murine GM-CSF caused division in the microglia, and these cells differentiated in response to 50ng/ml rhM-CSF. All of these factors are produced at least in part, by astrocytes, and it has therefore been proposed that CSFs regulate the number of microglia in the developing CNS. The work *in vitro* must be interpreted with caution since many studies have used inflammatory stimuli to activate astrocytes. Nevertheless, several key cytokines including M-CSF and GM-CSF have now been found *in situ* within the normal developing brain (Chang et al. 1994; Thery et al. 1990).

Other than direct interactions with astrocytes and their migratory response, further characteristic patterns of behaviour for human foetal microglia emerged from the co-culture studies presented in Chapter IV: when cultured microglia encountered debris, they emitted pseudopods that encircled, engulfed and ultimately assimilated the particles. Multipolar microglia whose lamellipodia projected in several directions appeared to explore the surrounding environment to orientate their movement. In addition, intermediate morphologies between multipolar, bipolar and unipolar types were observed in the same cell, indicative of interchangeable morphological states. Microglia were found to occupy distinct 'territories' and very rarely did they make contact. These observations further indicated that the patterns of microglia distribution and differentiation could be mimicked in culture. This is contrary to other cell types found in the CNS, and raises the possibility that microglia themselves can secrete factors that initiate interconversion and regulate their spatial distribution, both *in vitro* and *in vivo*. Which then, are the signals that can initiate the patterns of differentiation and migration of microglia that we can detect *in situ* in the developing brain?

Factors regulating microglial ramification: A number of factors are known to be associated with or induce microglial ramification (**Figure 101**). As noted, chief amongst these is the influence exerted by astrocytes. Both soluble (M-CSF: Hao et al. 1990; Liu et al. 1994; Sawada et al. 1990; GM-CSF: Fujita et al. 1996) and insoluble factors (Tanaka and Maeda, 1996) secreted from astrocytes have been known to cause microglial ramification and proliferation. Moreover, amoeboid microglia ramify on fixed or freshly cultured astrocyte monolayers (Lee et al. 1992; Tanaka and Maeda, 1996). This implies that a component or substrate (such as integrins) derived from the extracellular matrix and present on astrocytes is required for contact-mediated ramification of microglia (Giulian et al. 1995). Fibronectin, vitronectin, laminin, thrombospondin and tenascin have all been suggested as mediators (Chamak and Mallat, 1991; Smith and Hale, 1997; Tanaka and Maeda, 1996). Several studies

have supported this hypothesis: astrocyte-derived ECM components including laminin and fibronectin (as well as serum-free medium) can induce microglia to ramify (Fujita et al. 1996; Tanaka et al. 1998, 1999), although laminin may also promote the amoeboid form of microglia (Giulian et al. 1995). Similarly, blood monocytes and tissue macrophages ramify on astrocyte monolayers, but not on acellular substrates or on fibroblasts (Sievers et al. 1994), or on mesenchymal/epithelial cells (Wilms et al.1997). These cells downregulate their antigenic markers and display ion channel properties similar to those of microglia (Schmidtmayer et al. 1994).

As shown in this study, the ramification and motility of microglia depend on the state of confluency of astrocytes in culture. Microglia tended to adopt more stable morphological states and motility in progressively confluent cultures of astrocytes. Therefore the density of astrocytes in culture exerts a separate influence on microglial numbers and ramification (a plating density of less 8,000 cells/cm^3 inhibits the growth of microglia, which appear as isolated cells: Richardson et al. 1993; Sievers et al. 1994). According to another study, a plating density of less than 10,000 microglial cells per 24-well plate was found necessary for microglia to ramify (Heppner et al. 1998). Secretory products released by astrocytes clearly affect microglial morphology. As we have seen, GM-CSF and M-CSF produced by astrocytes *in vitro*, are not only potent stimulators of microglial proliferation and survival, but are also required for their morphological differentiation (Aloisi et al. 1992; Ganter et al. 1992; Giulian et al 1995; Giulian and Ingemann 1988; Lee et al. 1992, 1993, 1994; Liu et al. 1994; Malipiero et al. 1990; Suzumura et al. 1990, 1991). Liu and colleagues (1994) reported that an antibody to the M-CSF receptor effectively inhibited the ramification of microglia co-cultured with astrocytes. Microglia may be induced to ramify in serum-free medium supplemented with 2ng/ml GM-CSF (Fujita et al. 1996).

Serum supplements may also influence microglial morphology (Fujita et al. 1996). Constituent proteins within foetal calf serum (e.g. progesterone, insulin, casein) as well as high glucose concentrations can inhibit GM-CSF-induced ramification of microglia, whereas microglia that are cultured in media devoid of glycine and serine display a ramified morphology (Tanaka et al. 1998; Yao et al. 1990). Microglia cultured in serum-free medium on poly-L-lysine coated coverslips or in long-term tissue culture slice preparations likewise exhibit a ramified morphology (Czapiga and Colton, 1999; Mertsch et al. 2001; Sudo et al. 1998), and their macrophage-associated activities are suppressed, as indicated by a reduction in superoxide ions produced. Other factors such as thyroid hormones exert proliferative and differentiating effects that promote ramification of microglia in rodents (Lima et al. 2000). Therefore, certain constituents within serum can maintain microglia in an activated state. This

may be of particular relevance during early stages of development, when the blood-brain barrier is not completely formed, and amoeboid microglia could be maintained in their activated states at these sites through exposure to serum components. For comparison, in the adult, microglia also adopt a more activated state in areas of the CNS that lack a complete blood-brain barrier (Perry and Gordon, 1991).

A number of other determinants known to induce microglia to ramify have been reported. Treatment with antioxidants such as α-tocopherol and ascorbic acid, initiate microglial ramification within 48 hours with accompanying downregulation of surface antigens (LFA-1, VLA-4 and ICAM-1) and increased survival (Heppner et al. 1998). Thapsigarin, a calcium-ATPase inhibitor of endoplasmic reticulum has been reported to promote ramification *in vitro* (Yagi et al. 1999). Agents known to accelerate the differentiation of CNS precursor cells (e.g. retinoic acid and dimethylsulphoxide) also stimulate the formation of processes by microglia *in vitro*, of up to 700μm in length (Giulian and Baker, 1986; Yao et al. 1990). Nanogram amounts of bacterial lipopolysaccharide (LPS), reportedly also induces ramification although this usually predisposes microglia to activation and their associated amoeboid forms (Mertsch et al. 2001). Finally, an alteration in the levels of free radicals available to microglia may be influential. Scavenging of free radicals is a property that has been noted in microglia (Noble et al. 1994), and free radicals have the potential to activate and maintain microglial activation, particularly in isolated cultures devoid of other cell types that could act in some buffering capacity. Low serum levels (5% foetal calf serum, 1% normal human serum) in addition to mercaptoethanol (which inhibits superoxide and free radical) in this study may have contributed towards the progressive ramification of microglia in co-culture. Nonetheless, the most convincing requirement for microglial ramification appeared to be direct contact with astrocytes. On the whole, it is therefore likely that the ramification of microglia will depend on a combination of factors and on the level of contribution of each of these that are present in the milieu of the normal developing CNS.

Factors regulating microglial motility: Migratory cells respond to mutiple directory cues in a co-ordinated manner. To accomplish this, adhesion receptors such as integrins and cadherins that are expressed at the surface of cells, mediate interactions between the cell and ECM as well as direct cell-cell interactions necessary for migration (Huttenlocher et al. 1998). Within cells, the actin cytoskeleton provides the extension and contractile forces required for cellular movements (phagocytocis, cytokinesis, crawling and contraction) including migration using lamellipodia or pseudopodia (Stössel, 1993). This takes place through a combination of actin polymerisation, depolymerisation, cross-linking and interaction with myosin (Allen et al. 1998; Bray 1992). In macrophages, the regulation of these activities is partly governed by the

Rho family of GTPases: Rho, Rac, and cdc42 which activate the M-CSF tyrosine kinase receptor (Allen et al. 1998; Webb et al. 1996). These actin-regulatory proteins modulate cell migration during chemotaxis of macrophages *in vitro*. The dynamic protrusion and retraction of processes by microglia, movements of the cell body and internal structures noted in this study and by others, all indicate a flexible cytoplasm characteristic of macrophages, that can be inhibited by blocking actin polymerisation (Brockhaus et al. 1996). Examining slice cultures of corpus callosum from postnatal murine CNS, Brockhaus and colleagues noted that microglia within the slices retracted their processes and migrated at velocities of 0.5-1μm/minute (30-60μm/hour) to the surface of the slice where they projected vellum-like processes in a rotary motion. This compared well with speeds of around 25 to 75μm per hour recorded for human foetal microglia in this study. Rapid movement of these processes preceded the generation of phagocytic and pinocytic vesicles. In their investigation, migration followed a direct route or occurred indirectly after traversing several micrometers parallel to the surface. Pseudopods of these activated cells were involved in phagocytosis of dead or dying cells at the surface, and their movement could be inhibited by treatment with cytochalasin B (an inhibitor of actin). The motility of microglia can also be directly affected by changes in the extracellular pH: acidification reduces motility through bundling of f-actin and formation of stress fibers (Faff and Nolte, 2000).

It is also recognised that cells are further guided by the substratum over which they move. Insoluble guidance molecules promote or inhibit migration, and physical contours are also important. For example, when a cell encounters as fibre or filament, the ruffled membranes follow the fiber and pull the cells into a bipolar shape (Bray, 1992). Thicker fibers exert more powerful contact guidance than thin fibers: the shape of cells can be controlled by abundance of thicker fibers. Substratum guidance is particularly important for the migration of cells in developing embryos, where ECM components are known to regulate cell motility. For example laminin, a prominent ECM component of the CNS, has been shown to promote the migration of neuronal and oligodendrocyte precursors (Schmidt et al. 1997). Microglia preferentially associate with and migrate on astrocytic substrates, and laminin promotes their amoeboid 'migratory' phenotype in culture (Chamak and Mallat, 1991). Furthermore, microglia constitutively express the β2 integrins CD11a/LFA-1, CD11b/Mac-1/CR3, CD11c/CR4, and CD18 (Akiyama and McGeer, 1990), which are involved in interactions with ECM components and adhesion molecules within the CNS. These can be modulated *in vivo* and in culture and are associated with the morphological transformation and migratory response of microglia (Kloss et al. 2001).

In order for cells to migrate, the ECM has to be degraded enzymatically. In this regard, matrix metalloproteinases (MMPs) and other ECM-degrading enzymes are also of great significance, not only in migration, but also for the remodelling of tissues which is part of the process of maturation of the nervous system. Human microglia have been shown by others to express MMPs 1,2,3 and 9 in the adult brain (Maeda and Sobel, 1996), membrane-type MMP, an activator of MMP-2 (gelatinase-A) in the white matter (Yamada et al. 1995), as well as the receptor for urokinase plasminogen activator (an active cell surface protease) in cell culture (Washington et al. 1996). Migration is further directed by chemical cues within the environment of a cell (Haapaniemi et al. 1995). Microglia have been shown to respond by chemotaxis and migration to a variety of chemoattractive stimuli in the brain (Graeber et al. 1993; Nolte et al. 1996). These include β-amyloid peptide (Davis et al. 1992; Fiala et al. 1998; Maeda et al. 1997; Nakai et al. 1998), complement 5a (Faff and Nolte, 2000; Nolte et al. 1996; Yao et al. 1990); TGF-β (Yao et al. 1990), NGF (Glad and Gilad, 1995), LPS (Yang et al. 1996), neuronal platelet activating factor (Aihara et al. 2000), leukaemia inhibitory factor (Sugira et al. 2000), epidermal growth factor (Heppner et al. 1998; Nolte et al. 1997), neuronal M-CSF (duYan et al 1997), substance P (Maeda et al. 1997), zymosan-activated serum (Czapiga and Colton, 1999) and chemokines: C10 (Asensio et al. 1999), RANTES (Cross and Woodroofe, 1999; Hu et al. 2000), the beta chemokines MIP-1α, MIP-1β, MCP-1 (Cross and Woodroofe, 1999; Peterson et al. 1997), as well as IL-8, IP-10 (Hu et al. 2000), and neuronal fractalkine (Harrison et al. 1998; Pan et al. 1997). In particular, microglia can respond rapidly by migration to a site of injury or inflammation, where many of these stimuli accumulate. In contrast to astrocytes, microglia display a marked migratory response to the chemokines, particularly MCP-1 (Calvo et al. 1996; Hayashi et al. 1995), which in turn can induce the production of IL-1 and IL-6 in mononuclear cells (Jiang et al. 1992). Therefore these studies all suggest that a chemotactic gradient if present focally, can amplify the recruitment of microglia.

Microglial motility *in situ*: Colonisation of the CNS by microglia necessitates long distance migration of progenitors. This migration is brought about partly through the association with radial glia (Rakic 1981). For example, amoeboid microglia in the avascular embryonic retina migrate tangentially along Müller radial glia (Cuadros and Navascues, 1998; Navascues et al. 1996). This model is useful as it precludes complications of precursor influx from vessels. Tangential migration occurs along a tortuous pathway with cell movements in various directions. Amoeboid cells adhere strongly to Müller cell endfeet and processes at focal contacts and appear to migrate along these. Vellum-like lamellipodia have been noted to emerge from the cell body, from short and thick, or long and delicate processes. Although these lamellipodia from neighbouring cells do occasionally make contact, they do not overlap.

which suggests that contact-inhibitory factors are at play (Bershadsky and Vasiliev, 1988). Microglia are oriented along and generally migrate from central (optic disc) regions to the periphery of the retina, although migration is not entirely unidirectional. Again, this behaviour appears to be similar to that of cells migrating along a gradient of chemoattractant (Yao et al. 1990).

Axonal tracts are the preferred sites for migration of glial progenitors. For example, during development, O2A progenitor cells specifically invade and associate with axonal tracts, where they differentiate into oligodendrocytes, extending their processes between fascicles and synthesise myelin. The corpus callosum is a major axonal tract that developmentally contains both microglia and oligodendrocyte precursors. Microglia accumulate in great numbers at the surface of tissue slice preparations, and in some cases, migrate parallel to the fibers of the corpus callosum (Brockhaus et al. 1993). However, the direction of most motile cells is towards the cortex, with more than 80% of motile cells traversing parallel fiber tracks of the corpus callosum (Cuadros and Navascues, 1998). Similarly, amoeboid microglia appear to migrate along axon fascicles within the developing optic nerve (Moujahid et al. 1997), and cerebellar white matter (Navascues et al. 1996; Cuadros et al. 1997). Myelinated tracts appear to be a favoured site for the migration of other cell types including transplanted cells (for example xenogeneic astrocytes, Booss et al. 1991) in the absence of an inflammatory response. Mechanical guidance through adhesion molecules, such as NCAM, laminin, or heparan sulphate proteoglycan are likely to be involved in this type of migration. Both the depth and interspacing of fascicles may influence the degree of alignment of cells, as demonstrated by parallel alignment of cells on a corrugated surface in culture (Bray, 1992). In a similar fashion grooves between fascicles may therefore provide highways for neuronal growth cones and associated microglia.

Factors regulating microglial proliferation and apoptosis: The behaviour of astrocytes in long-term cultures can be explained partially by observations of the behaviour of other cell types, where cells that are proliferating will come into collision more frequently, and cell migration between contacts is shorter. Eventually, when confluence is reached, cell movement stops. If cells are not subject to contact inhibition, they will often overly others in culture (Bray, 1992). This is typical of human foetal astrocytes in culture. However, evidence suggests that the upper surface of confluent cells exposed to the medium, does not provide a sufficiently adhesive surface for other cells to migrate over. Instead, the layering results from one cell crawling under another (Bray 1992). This phenomenon is typical of microglia in long-term co-cultures with astrocytes, where these cells can be seen to roam under the confluent layer of astrocytes. Early studies with cultured microglia showed that 'epithelioid cells'

(astrocytes) eventually dominated the culture and thereafter, microglia rapidly died (Dunning and Furth, 1935). In Chapter IV we also saw that microglia overlying astrocytes in long-term co-cultures progressively detached and subsequently underwent a form of cellular death. These processes are likely to be elicited either due to factors that accumulate within the supernatant (Mattion et al. 1995), or as a result of the non-permissive nature of astrocytes once they have established contacts and formed confluent sheets. In this respect, a lack of growth or related factors may be partly responsible for microglial detachment. Colony-stimulating factors such as G-CSF, GM-CSF, M-CSF and IL-3 are recognised as growth promoters for cells of the mononuclear phagocyte lineage (Metcalf, 1985; Nicola, 1989). GM-CSF and M-CSF are also growth factors for amoeboid microglia (Blevins and Fedoroff, 1995; Giulian and Ingemann, 1988; Lee et al. 1994; Sawada et al. 1990), and are produced by astrocytes (Lee et al. 1993; Malipiero et al. 1990; Ohno et al. 1990; Thery et al. 1990) and neurons (Nohava et al. 1992). Withdrawal of these mitogens *in vitro* leads to an elevated rate of microglial cells death via DNA fragmentation (Gehrmann, 1995). For example, microglia undergo apoptosis characterised by the shrinkage of cytoplasm, nuclear condensation and/or fragmentation, forming apoptotic bodies when M-CSF is deprived from the culture medium (Tomozawa et al. 1996), and this can be detected by *in situ* nick-end labelling of fragmented DNA (Gavrielli et al. 1992). Contrary to astrocytes, human foetal microglia (and neurons) readily undergo apoptosis in tissue culture, when exposed to the opiate receptor agonist morphine (Hu et al. 2002). This property is also consistent with studies of apoptosis following the exposure of rodent peritoneal macrophages and human blood monocytes to morphine (Singhal et al. 1998, 2000). In Chapter IV, it was further noted that human foetal microglia (and astrocytes) exposed to recombinant MCP-1 at [100ng/ml] for several hours, also showed nuclear alterations characteristic of cells undergoing apoptosis, with fragmentation of DNA evident towards terminal stages. TUNEL labelling could not be reliably performed on these cultures, since the majority of these cells were disrupted morphologically upon fixation. Therefore this seemingly important finding, that long exposure to high concentrations of MCP-1 may actually induce apoptosis in human foetal microglia and astrocytes, will have to be verified in independent studies.

Although microglial proliferation has been extensively studied in disease states, following trauma, or injury to the CNS, less attention has been directed towards the developing brain (Giulian et al. 1991; Lee et al. 1994). Many of the macrophages within the developing CNS are believed to undergo apoptosis and will not survive to adulthood, while others are capable of proliferating prior to differentiation. Here, using dual-label immunohistochemistry with CD45:CD68 and PCNA, it has been estimated that up to one third of microglia within the intermediate zones are undergoing division during the second trimester. Notably, despite the

abundant cell division noted at various sites in the cerebrum (including the ventricular zone and choroid plexus), only microglia appeared to express PCNA within the intermediate zone. Microglial proliferation therefore contributes to the process of colonisation of the developing nervous system

Various studies indicate that M-CSF, GM-CSF, IL-3 produced by foetal astrocytes and microglia (Blevins and Fedoroff, 1995; Giulian and Ingemann 1988; Lee et al. 1992, 1994; Liu et al. 1994; Raivich et al. 1994; Sawada et al. 1990; Schlichter et al. 1996; Tanaka and Maeda, 1996) or adenosine (Gebicke-Haerter et al. 1996) may act as possible signals for microglial division, whereas deprivation may lead to apoptosis. More specifically, *in vitro* studies indicate that either GM-CSF (Lee et al. 1994) or GM-CSF like peptides in rodents (Giulian et al. 1991), are responsible for microglial proliferation during embryogenesis, whereas the role of M-CSF is more controversial. All of these factors are produced at least in part, by astrocytes, and it has therefore been proposed that CSFs regulate the number of microglia in the developing CNS. Several key cytokines including M-CSF have been found within the normal developing mouse brain (Thery et al. 1990). Chang and colleagues (1994) showed that M-CSF (but not GM-CSF or IL-3) mRNA was constitutively synthesised from E13, with particularly high levels from E17 to postnatal day 15, and diminishing thereon through to adulthood. This corresponds to the period when microglia are proliferating and differentiating to ramified forms in the mouse brain, as shown in Chapter III, following which amoeboid cells are noted to decline in numbers. M-CSF appears to affect both the survival and differentiation of microglia. Tomozawa and co-workers (1996) found that 1ng/ml of recombinant human M-CSF enhanced the survival rate of rodent microglia in culture. They also found that 5ng/ml rhM-CSF or 1ng/ml recombinant murine GM-CSF caused division in the microglia, and these cells differentiated in response to 50ng/ml rhM-CSF. Furthermore, microglia underwent apoptosis associated with extensive nuclear condensation and fragmentation, within 48 hours following deprivation of M-CSF. These authors correlated their findings with the disappearance of a large proportion of amoeboid cells by about the 15^{th} postnatal day in rodents, following the completion of synaptogenesis. In mice genetically deficient in M-CSF (osteopetrotic mutants), although microglia are present within the CNS in appreciable numbers (60-70% compared to wild-type animals), their proliferative capacity is reduced by as much as 90% as assessed by the facial nerve transection model (Blevins and Fedoroff, 1995; Wegiel et al. 1998; Wiktor-Jedrzejczak et al. 1991; 1996; Witmer-Pack et al. 1993). These studies highlight the importance of both M-CSF and GM-CSF on the correct proliferation and differentiation of microglia during the foetal period.

Electrophysiological properties specific to microglia

This study further showed that the electrophysiological property of microglia *in vitro,* was not altered by the length of cultivation (as opposed to freshly isolated cells). was independent of the morphological state of microglia, and consistent between species (rodent and human). Rodent and human microglia possessed membrane potentials around -70 to -80mV. If clamped at -70mV, they showed no currents when subjected to a hyperpolarising voltage step, but for depolarising voltage steps, a large inward-rectifying current was detected. Potassium channel blocking agents (such as TEA, 4-AP and barium) are known to block this inwardly rectifying current. The lack of outward currents is believed to render microglia sensitive to depolarising events, which are long-lasting and may allow the transition of microglia from one morphological state to another. This depolarisation can be induced by exposure to ATP. Recently, Boucsein et al. (2000) have confirmed that the inward-rectifying K+channel is also a marker for activation and possibly triggering of microglia *in vivo.*

However, this property seen in activated microglia is not inherent in resting ramified cells *in situ.* Within organotypic tissue slice preparations of the rodent CNS, ramified (perivascular) microglia are instead characterised by little if any inward voltage-gated membrane currents, and very low membrane potentials (their resting potential is closer to -20mV rather than -70mV). Microglia in these slices, exhibit larger, passive conductance over the entire stepping voltage range, and it is likely that their resting conductance is due to non-specific cationic conductance (Boucsein et al. 2000). Despite this characteristic, once these cells are activated (as in the facial nucleus, following transection), their activation parallels an increase in membrane area and a shift to a more negative potential, due to an increase in K+ conductance compared with non-specific cationic conductance. That is, they resemble isolated and cultured microglia. Furthermore, the inward current appears to be a transient phenomenon, since it can be identified within 12 hours following axotomy (in the facial nerve transection model). Within 24 hours, microglia express a prominent outward current (usually seen following LPS-activation of microglia *in vitro*), and by one week following the lesion, their 'resting' electrophysiological properties have been regained, in parallel with the morphological transformations and with the ensuing reparative processes that are underway within the tissue at this time.

Thus the inward rectifying potassium current can be taken as an early marker (or trigger) for microglial activation *in vivo.* It remains to be seen whether ramified *human* microglia overlying confluent astrocytes, and amoeboid and early ramified microglia *in situ* in the developing human CNS, will adopt the electrophysiological characteristics typified of rodent microglia seen under these experimental conditions. The specific question would be whether

amoeboid microglia similarly downmodulate their potassium currents upon differentiation to early ramified forms. Likewise, it would be important to determine whether potential populations of microglial progenitors residing in cord blood or within foetal haematopoietic tissues (yolk sac, spleen and liver), could be identified by these electrophysiological characteristics, and how these properties are affected if these progenitors are stimulated to differentiate to form microglia, for example by co-culture with human foetal astrocytes.

Cerebrovascular development and expression of adhesion molecules

Cerebrovascular development and differentiation begin with the formation of the neural tube and progress throughout gestation. By the end of the 4th week, the human embryo has a well-developed vascular system and its heart is beating. Vessels arise from mesodermal cells independently in the yolk sac, the chorion and the embryo. These three systems of vessels later unite to establish the complete circulation. Vascularisation of the CNS commences at 5 weeks near the leptomeningeal side of the neural plate and between the 5th and 6th weeks, numerous yolk sac-derived blood cells occur within and outside blood vessels in human embryos (Choi, 1981; Kershman 1939). Within the mature CNS, endothelial cells are characterised by continuous 'tight junctions' resulting in a high electrical resistance which contributes to the blood-brain barrier. This is induced in the CNS by the neural environment during development, and principally by astrocytic contact as already outlined. From studies conducted in rodent and chick embryos, the timing of barrier formation (based on impermeability to inert tracers) is known to depend on the anatomical location of blood vessels: a barrier in the spinal cord forms earlier than in the telencephalon and probably commences around E13 in the chick and E16 in rodent embryos. Although the precise timing of formation of the blood-brain barrier has not been determined in humans, indirect evidence suggests that tight junctions develop from the 7th to the 20th gestational weeks (Møllgard and Saunders, 1986).

Figure 102 shows a general schema for the development of blood vessels in the cortical plate of the telencephalic neocortex. From the earliest time points examined, microglia displayed a close relationship with the development and maturation of the cerebrovasculature. The appearance of microglial progenitors coincided with vascularisation of a particular region. In particular, human foetal microglia and their potential (amoeboid) progenitors were closely and preferentially associated with certain radiating cortical vessels in the telencephalon, capillaries within the germinal layers and corpus callosum, and with blood vessels in the internal capsule and sites bordering the caudate, as well as within the germinal matrix, basal ganglia and thalamus.

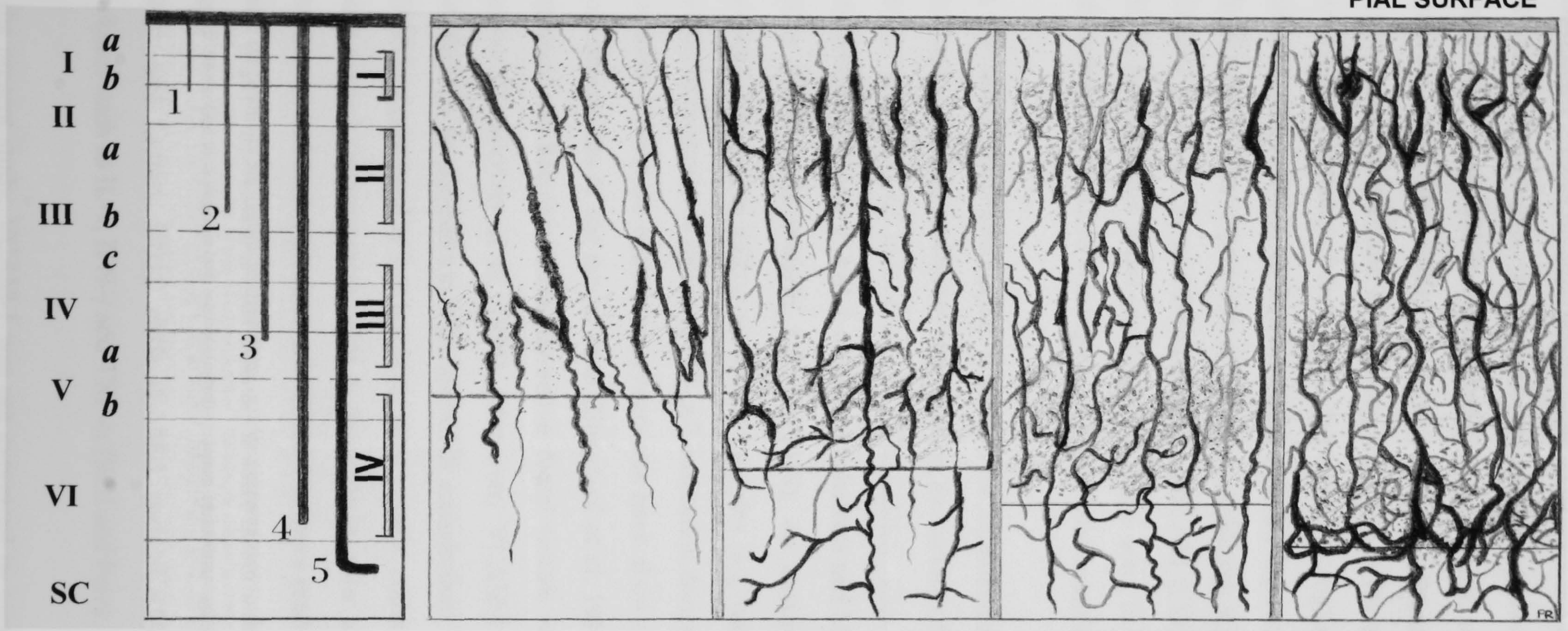


FIGURE 102

Vascular organisation of the human foetal cerebral cortex. Adapted from Duvernoy et al. 1981; Markovic and Marinkovic 1991; Norman and O'Kusky 1986. The degree of intracortical blood vessel penetration into the adult cortex is shown to the left of the figure. 1-5 represent five groups of intracortical arteries and veins. [I] molecular layer, [II] external granular layer, [III] pyramidal layer, [IV] internal granular layer, [V] ganglionic layer, [VI] multiform layer of cortex. Four vascular layers, shown on the right [I-IV] can be identified in the adult, based on regional differences in relative densities of capillary bed formation. The greatest vascular density of the cortex is within the third vascular layer. SC: subcortical region. Development of foetal cerebrovasculature is presented to the right. Between 15 and 21GW, blood vessels penetrate the nervous tissue in a regular geometric pattern. The cerebral cortex is supplied by vessels that arise from pial arteries. At 15GW, intracortical vessels are radially oriented and originate from the leptomeninges. Most of these radial vessels course through the cerebral cortex without branching to vascularise the subcortical tissue. By 20GW horizontal branches arise from radial vessels, most frequently in the lower half of the cortex, with occasional recurrent collaterals ascending to more superficial layers. From 20-27GW, the number of horizontal branches and recurrent collaterals increase in the lower half of the cortex, and horizontal branches appear in the upper half. Radial vessels of smaller lengths progressively increase with gestation in the cortical laminae, with the eventual network formation of capillaries after birth.

The finding that foetal microglia preferentially associated with certain large-diameter blood vessels (for example within the internal capsule) is intriguing. It raises the possibility whether they can use such vessels for migration to various sites. Certainly, time-lapse video microscopic studies on slice preparations from the CNS of postnatal rodents, have found that perivascular microglia can indeed migrate along cerebral vessels during development (Grossmann et al. 2002). Alternatively, it is possible, although not confirmed, that factors associated with the walls of these microglia-adherent blood vessels, stimulate these progenitors to divide, and in so doing, may add to the local population of foetal microglia. Nevertheless, in many of the regions displaying microglial progenitors (particularly amoeboid cells), there was evidence for circulating cells having recently gained entry into the CNS via blood vessels. The expression of adhesion molecules ICAM-1 (CD54), VCAM-1 (CD106), ICAM-2 (CD102), E- and P-selectin, and PECAM (CD31) were examined between 16 and 23GW in the human foetal cerebrum and spinal cord, in order to identify whether microglial infiltration into the CNS was regulated by regional vascular expression of these molecules.

In the adult, trans-endothelial migration of mononuclear phagocytes occurs via specific binding to adhesion molecules expressed on the surface of vascular endothelium. Observations on the migration of monocytes to inflammatory sites have indicated the importance of interactions between ICAM-1 on the endothelium and LFA-1 ($\alpha_L\beta_2$ integrin, CD11a/CD18) and/or CR3 ($\alpha_M\beta_2$ integrin, CD11b/CD18), on monocytes and macrophages. VCAM-1 mediates adhesion to leukocytes via α4 integrins (e.g. $\alpha4\beta1$ and $\alpha4\beta7$) (Shimizu et al. 1992) and has been shown to facilitate cell-cell interaction during development (Gurtner et al. 1995). However, studies on human embryogenesis have shown that VCAM-1 is not expressed on most derivatives of the neural crest (Sheppard et al. 1994). Instead, VCAM-1 is selectively expressed on haematopoietically-active foetal tissues such as the spleen, liver and bone marrow (Schweitzer et al. 1996). Moreover, VCAM-1 is not readily inducible on CNS endothelium in comparison with non-CNS endothelium (dos Santos et al. 1996). Although ICAM-1 is expressed at low levels on most vessels, endothelial cells do not express VCAM-1 under normal circumstances. This is desirable in order to limit the trafficking of leukocytes into tissues. Induced expression of VCAM-1 is more readily detected in postcapillary venules, a site of prominent leakage and leukocyte extravasation during inflammation, though VCAM-1 can also be encountered on capillary endothelium and arterioles at sites of inflammation (Pober and Cotran, 1991). Both ICAM-1 and VCAM-1 are inducible by inflammatory cytokines such as IL-1, IFN-γ and TNF-α (Bell and Perry, 1995).

In the present study, VCAM-1 was not expressed by cerebral vascular endothelium between 16 and 23GW at a time when microglia are colonising the CNS. This therefore precludes the

use of VCAM-1 for recruitment of these cells. Similarly, the expression of ICAM-1 was largely absent in normal foetal brains, as reported for the adult brain (Kupner et al. 1990). ICAM-1 was found to be expressed sparsely by foetal cerebral vessels in this study. The intensity of the stain was weak and the distribution did not correlate with zones of microglial influx. Furthermore, there was no evidence for the expression of VCAM-1 or ICAM-1 in the human foetal spinal cord. The finding that ICAM-1 and VCAM-1 were similarly not expressed in the murine foetal CNS contradicts the findings of Dalmau and colleagues (1997). who found expression of ICAM-1 in the developing rat brain and postulated that it may contribute to recruitment of microglial progenitors. However, it does make sense from the viewpoint that events, which occur during embryonic and foetal development. are quite distinct from those that preside in inflammation (Heyward et al. 1995). In this respect. P-selectin and E-selectin, known to be important contributors to the interaction of monocytes with venular endothelium during inflammation (Bevilacqua 1993), were also found to be largely lacking from the developing CNS in this study.

An alternative ligand for LFA-1 (and probably also for Mac-1) is ICAM-2, and this adhesion molecule is non-inducible by inflammatory cytokines (Acevedo et al. 1993; Holness et al. 1995; Nortamo et al. 1991; Staunton et al. 1989), but does seem to be specifically expressed on endothelium at varying levels in different tissues (Ebnet et al. 1996). For example, low levels of ICAM-2 are constitutively expressed by human brain microvessel endothelium isolated in culture (Brayton et al. 1998), and on a human bone marrow endothelial cell line (Schweitzer et al. 1997). Similarly, the levels of ICAM-2 expression on adult brain endothelium are reportedly low (Navratil et al. 1997), which may partly explain why lymphocyte trafficking into the CNS is limited under normal circumstances. Current opinion regards the expression of ICAM-2 on endothelium as necessary but not sufficient to induce monocyte migration. Additional signalling molecules (such as MCP-1) are also required. Unexpectedly, this investigation showed a relationship between vascular ICAM-2 expression and highly vascularised areas of the human brain colonised by microglia, particularly within the corpus callosum at 16GW and more widespread on radiating cortical vessels by 22GW. Similarly, ICAM-2 was present on the vasculature in the connective tissues surrounding the spinal cord between 9 and 16GW, and these areas correlated with macrophage entry. Within the cord, ICAM-2 positive vessels were first detected around 12 weeks and more prominently by 16 weeks. The majority of these vessels were located in the dorsal and ventral funiculi, extending into the mantle layer. which corresponded to the pattern of migration for foetal microglia that took place from 14-16GW, and was directed inwards from the connective tissue surrounding the spinal cord both dorsally and ventrally. These findings suggest that an interaction between LFA-1/Mac-1 and ICAM-2 is at least partially required for the migration

of microglial progenitors. This would seem sensible from the point of view that ICAM-2 is developmentally expressed and subsequently downregulated on adult CNS endothelium. The finding that ICAM-2 was not expressed in the murine CNS between E15 and birth may suggest a species-specific and/or functional specialisation in the developmental expression of this vascular adhesion molecule. Nevertheless, the expression of ICAM-2, although necessary is probably not sufficient to induce the migration and recruitment of monocytes alone.

In the present study, both PECAM-1 and laminin were highly expressed on the majority of foetal cerebral vessels irrespective of localisation with microglia, including on capillaries within the germinal layers between 16 and 22GW, and within the spinal cord between 9 and 16GW. The expression of subunits of laminin on cerebral blood vessels has been associated with their development. For example in rodents, S-laminin has been detected specifically on developing cerebral vessels during the perinatal period, and coincides with the maturation of the blood-brain barrier, whereas laminin is constitutively present on the walls of vessels throughout development and in the adult (Hunter et al. 1992). In the developing spinal cord of rats, Hunter and colleagues (1992) further found laminin to be present throughout the pia, whereas s-laminin was concentrated specifically on the pia that overlay the floor plate. This region has been proposed to provide extracellular cues for guidance of growing axons, and as shown by the present study, also accumulates microglial progenitors. It should therefore be determined whether microglial progenitors specifically interact with specific subunits or isoforms of laminin and whether these molecules are developmentally expressed in a region-specific manner within the human foetal CNS.

PECAM-1 (CD31), a 120-130Kd membrane glycoprotein expressed on endothelial cells, platelets and a variety of mature haematopoietic cells including monocytes, neutrophils and subsets of T cells (Watt et al. 1995), mediates transendothelial migration of leukocytes by heterophilic (glycosaminoglycan-dependent) or homophilic (glycosaminoglycan-independent) mechanisms (Baldwin et al. 1994; Yan et al. 1995). It is abundantly expressed on endothelium both *in vivo* and in culture (Muller et al. 1989). In the adult, PECAM-1 is constitutively expressed on endothelial cells and preferentially localised at sites of cell-cell contact. This restricted pattern of expression is considered to contribute towards interactions that limit vascular permeability. This is also particularly significant to cell migration since antibodies raised against CD31 block normal cell-cell contact and disrupt cell migration (Schimmenti et al. 1992). Experiments also suggest that homophilic interaction between leukocyte and endothelial CD31 molecules result in signal transduction that consequently increases adhesion mediated by β1 and β2 integrins (or in other words, via CD11/CD18 or α4β1 dependent mechanisms) (Bevilacqua 1993). PECAM-1 can potentially induce integrin-modulated

adhesion at high endothelial venules (Shimizu et al. 1992). PECAM-1 has also been suggested as a useful marker for following vascular formation in mammalian embryos. It has been localised to early endothelial precursors within the yolk sac and subsequently in all organs and tissues within the embryo (Baldwin et al. 1994; Vecchi et al. 1994), and is expressed at high levels on all early myeloid cells (Lund and Terstappen, 1993). The immunoglobulin domains of PECAM-1 (domains 1 and 2) are known to be involved in distinct processes of diapedesis and migration of cells through the extracellular domain (domain 6) (Liao et al. 1995). In addition, PECAM may contribute to endothelial barrier function *in vivo*, disruption leading to leakage of hepatic and renal blood vessels in mice (Ferrero et al. 1995). The constitutively high expression of PECAM between 16 and 22GW may therefore reflect a dual role for PECAM during development, namely a contribution to the migration of microglial progenitors into the parenchyma and the formation of a preliminary barrier.

Therefore, the expression of ICAM-2 on cerebral endothelium during the second trimester, associated most closely with the influx and distribution of foetal microglia. ICAM-2 is normally expressed at low levels in the adult brain and is not induced by inflammatory cytokines. Therefore, its association with microglial colonisation suggests tissue-specific control of this process, mediated by the endothelium. These findings can be placed in perspective when considering stages in development of the vascular system and of mononuclear phagocytes. In the early phases, mesodermal (phagocytic) cells gain access to the CNS prior to vascularisation. Following this, blood vessels develop within this tissue, and express ICAM-2 at certain distinct locations. Shortly afterwards, progenitor cells develop that have the capacity to interact with ICAM-2 and migrate into vascularised sites.

Expression of chemokines and chemokine receptors in the foetal brain and spinal cord

Cells of the human CNS are known to express or can be induced to express an expanding number of chemokines and their receptors. **Table 17** provides a summary of chemokines produced by human astrocytes, neurons and mononuclear phagocytes. **Table 18** lists the expression of chemokine receptors characterised in cells from the human nervous system. Certain chemokines may regulate neuronal functions such as synaptic transmission, enhance neuronal survival, and possibly contribute towards neuronal repair following injury, with additional support derived from glial cells (Araujo and Cotman, 1993; Bacon and Harrison, 2000; Brenneman et al. 1999; Giovanelli et al. 1998; Meucci et al. 1998; Ragozzino et al. 1998). Furthermore, neurons have been shown to express specific chemokine receptors (e.g. CCR2, CCR3, CXCR2, CXCR3, CXCR4) (Hesselgesser et al. 1997; Horuk et al. 1997; Zhang et al. 1998), and chemokines have been proposed to act as signalling molecules for neurons within specific 'chemokinergic' pathways such as that within the hypothalamo-

neurohypophyseal axis (Lira 1996; Sakamoto et al. 1996; Tani et al. 1996). Several lines of evidence have further shown chemokines to be important in directing neuronal migration both *in vivo* (Bagri et al. 2002; Hesselgesser et al. 1998; Horuk 1998; Klein et al. 2001; Ma et al. 1998; Zou et al. 1998) and *in vitro* (Bolin et al. 1998; Hesselgesser et al. 1997; Lazarini et al. 2000). Some studies have shown that chemokines may act as proliferating signals and migrational cues for glial progenitors (Dorf et al. 2000). Chemokines such as MIP-1α, MIP-1β, MCP-1, RANTES, IL-8, IP-10, SDF-1 and fractalkine are potent chemoattractants for microglia *in vitro* (Cross and Woodroofe, 1999; Harrison et al. 1998; Heesen et al. 1996; Hu et al. 2000; McManus et al. 2000; Pan et al. 1997; Peterson et al. 1997; Tanabe et al. 1997). and MIP-1α has some chemotactic effect on astrocyte progenitors (Tanabe et al. 1997). Microglia in particular display marked migratory responses to MCP-1, which can be produced by both cell types *in situ* (Calvo et al. 1996; Hayashi et al. 1995). Based partly on these previous findings, it was proposed that the migration of microglial progenitors in the CNS during development, was likely to be initiated by the establishment of intracerebral gradients of chemotactic agents such as chemokines.

In order to ascertain which chemokines were present in the developing human brain during the second trimester, the patterns of expression for the β-chemokines MCP-1, MCP-3, MIP-1α, MIP-1β, RANTES, the α-chemokines IL-8, IP-10 SDF-1, and the CX_3C chemokine fractalkine, were examined in the developing brain. It was found that MCP-1 localised to the subplate and lower cortical plate of the telencephalon, to projections of the corpus callosum, within the matrix of the ventricular and subventricular zones, and was additionally found in the choroid plexus. Importantly, the expression of MCP-1 was limited to the lateral regions of the cerebrum, and co-localised with accumulation of differentiating microglia in the subplate. Although astrocytes are known to produce MCP-1 from tissue culture studies, expression of this chemokine did not appear to co-localise with GFAP, but was more likely to be neuronal. Expression of MCP-1 in the cortex can be induced in hypoxic-ischaemia (for example after transient middle cerebral artery occlusion) (Wang et al. 1995). However, the fact that none of the normal human foetal tissues analysed showed pathological signs of hypoxic-ischaemic injury, coupled to the developmentally-restricted pattern of expression for MCP-1 found within the CNS, precluded expression of this chemokine to be the result of secondary phenomena. The expression of RANTES was more widespread on cells within the intermediate zone, progressing toward the cortical plate, whereas IL-8 was associated with radial cortical vessels of the frontal cortex (these were previously characterised to be PECAM and ICAM-2 positive), and to some extent within the meninges overlying the cortical plate. Unlike MCP-1, MIP-1α was not expressed within the cortical plate. Instead, this chemokine was found on cells within the choroid plexus, ventricular zone and intermediate zone.

Table 17. Summary of chemokine production by human microglia, astrocytes, neurons, monocytes and macrophages

Chemokine	Species	Stimulus	Method	Source
IL-8	Human foetal microglia	LPS, IL-1β, TNFα	Culture	Ehrlich et al., 1998a,b; Lipovsky et al., 1998
RANTES	Human foetal microglia	LPS, IL-1β, TNFα	Culture	Hu et al., 1999
RANTES, MIP	Human foetal microglia	IFNβ	Culture	McManus et al., 2000a
RANTES; MIP-1α	Brain macrophages/microglia	HIV encephalitis	In vivo/ culture	Sanders et al., 1998
I-309	Human foetal microglia	IFN-β/γ	Culture	Hua and Lee, 2000
RANTES, IP-10	Human foetal microglia	LPS	Culture	Hua and Lee, 2000
MIP-1α, MIP-1β	Human foetal microglia	IFNβ	Culture	Hua and Lee, 2000
MIP-1α, MIP-1β, RANTES	Human macrophages	HIV-1	Culture	Mayer and Schmidtmayerova 1997
MIP-1α, MIP-1β	Human monocytes	HIV-1	Culture	Schmidtmayerova et al., 1996
MCP-1	Human foetal astrocytes	IFNγ	Culture	McManus et al., 2000a
MCP-1	Human astrocytes	—		Barna et al., 1994; Gourmala et al., 1997
MCP-1	Human astrocytes cocultured with ECs	HIV-1 tat	Co-culture	Weiss et al., 1999
MCP-1	Human astrocytes	Inflammatory stimuli (TNFα, TGFβ)	Culture	Barna et al., 1994; Hurwitz et al., 1995
MCP-1	Reactive/hypertrophic astrocytes	Multiple Sclerosis CNS	In vivo	Van der Voorn et al., 1999
MCP-1	Astrocytoma	IFNγ	Culture	Zhou et al., 1998
MCP-1, IL-8	Human foetal astrocytes	Hypoxia	Culture	Zhang et al., 2000
MCP-1, RANTES	Astrocytes		In vivo/ culture	Brosnan et al., 1993; Noe et al., 1996; Ransohoff et al., 1996
MCP-1, RANTES, IL-8	Astrocytes, astrocytoma	IL-1, TNFα	Culture	Aloisi et al., 1992; Barna et al., 1994; Barnes et al., 1996; Desbaillets et al., 1997
MCP-1, RANTES, IL-8, IP-10	Primary human astrocytes	IL-1β, TNFα	Culture	Oh et al., 1999
MCP-1, IP-10	Human Astrocytes	Induced	Culture	Sanders et al., 1998
IP-10	Primary human astrocytes	IFNγ	Culture	Oh et al., 1999
IP-10, RANTES	Human foetal astrocytes	IL-1β + IFNβ/γ	Culture	Hua and Lee, 2000
RANTES	Human foetal astrocytes	IL-1β, TNFα	Culture	Hu et al., 1999
RANTES	Isolated human astrocytes	TNFα/ IFNγ	Culture	Janabi et al., 1999
RANTES, IL-8	Human foetal astrocytes	HIV transfection	Culture	Cota et al., 2000
MIP-1α, MIP-1β, MCP-1, RANTES	Human foetal astrocytes	LPS, IFNγ, TNFα	Culture	Xia et al., 1998
MIP-1α, MIP-1β	Isolated human astrocytes	IL-1β, TNFα	Culture	Janabi et al., 1999
MIP-1α	Human foetal astrocytes and microglia	IL-1β	Culture	Miyamoto and Kim, 1999
MIP-1α, MIP-1β, MCP-1	Human foetal astrocytes and microglia	LPS, TNFα, IL-1β	Culture	Peterson et al., 1997; Mcmanus et al., 1998
MIP-1α, MIP-1β, IL-8, GRO-α	Isolated human astrocytes & microglia	IL-1β, TNFα	Culture	Janabi et al., 1999
MIP-1α, RANTES	Human mixed CNS cells	TNFα	Co-culture	Lokensgard et al., 1997
Fractalkine	Neurons	Olfactory bulb, cerebral cortex, hippocampus, caudate, putamen, nucleus accumbens	In vivo	Harrison et al., 1998 (rat); Nishiyori et al., 1998; Schwaeble et al., 1998; Tong et al., 2000
IL-8	Hippocampus and cerebellar neurons		In vivo	Rothwell et al., 1990
MCP-1	Human foetal neurons	Cerebellum and brainstem (inferior olive, basal pontine nuclei)	In vivo	Meng et al., 1999
MCP-1	Differentiated human NT2 neurons	—	Culture	Coughlan et al., 2000

Table 18. Summary of chemokine receptor expression by cells of the human CNS

Chemokine receptor	Species	Source
CCR2	Human foetal astrocytes	Andjelkovic et al. 2002; Rezaie et al. 2002
CCR3, CCR5	Human astrocytes (multiple sclerosis)	Simpson et al., 2000
CCR5	Human astrocytes (hippocampus, cerebellum)	Rottman et al., 1997
CCR5, CXCR4	Human foetal astrocytes	Klein et al., 1999
CCR5, CXCR4 mRNA	Human foetal astrocytes	Sabri et al., 1999
CXCR2	Human foetal astrocytes	Hesselgesser et al., 1997
CXCR1, CXCR2, CCR2b	Human foetal astrocytes (HIV transfected)	Cota et al., 2000
CCR3, CCR5, CXCR4	Human microglia	Albright et al., 1999; He et al., 1997; Shieh et al., 1998
CCR3, CCR5, CXCR4	Human foetal CNS cells/ microglia	Gabuzda et al., 1999; He et al., 1997; Vallat et al., 1998
CCR3, CXCR4	Human CNS/PNS neurons	Zhang et al., 1998
CCR2, CXCR2; CXCR3, CXCR4	Human foetal neurons, hNT2 neurons	Coughlan et al., 2000
CCR1, CCR5, CXCR2, CXCR4	hNT neuronal cell body and processes	Halks-Miller et al., 1997; Hesselgesser et al., 1997
CCR5	Neurons (Limbic and associated areas)	Rottman et al., 1997
CXCR4	Neurons (Hippocampus, cerebellar dentate gyrus, amygdala, cingulated gyrus, thalamus)	Gabuzda et al., 1998; Lavi et al., 1997; Vallat et al., 1998
CCR3, CCR5, CXCR4	Neurons (Hippocampal, neocortical pyramidal, human foetal, adult and macaque monkey)	Lavi et al., 1998; Klein et al., 1999; Westmoreland et al., 1998
CXCR2	Human projection neurons (Hippocampus, dentate nucleus of cerebellum, pontine nuclei, locus coeruleus, paraventricular nuclei, Clarke'd column of spinal cord)	Horuk et al., 1997
CXCR2	Human foetal neurons (17-22 gestational weeks)	Hesselgesser et al., 1997
CXCR2	Human foetal neurons/ hNT cells	Pleasure et al., 1992; Halks-Miller et al., 1997
CX_3CR1	Primary CNS cultures (neurons, astrocytes, microglia)	Harrison et al., 1998; Jiang et al., 1998; Maciejewski-Lenoir et al., 1999; Meucci et al., 1998

Double-labelling with an antibody to CD68 revealed that MIP-1α positive cells in the intermediate zone were microglia. Notably, these cells commonly appeared in pairs. Paired microglial cells within this region also expressed PCNA, implying recent division. It is likely that within the ventricular zone, both MIP-1α and MCP-1 additionally labelled blood vessels. *In situ* hybridisation analysis also detected the mRNA for MIP-1α and MCP-1 confined to the ventricular zone, germinal matrix of the ganglionic eminence and more diffusely within the cortical plate. Taken together, these observations indicate that microglial progenitors could respond to differentially expressed chemokine gradients (MCP-1 and RANTES) by migrating away from the germinal layers towards the cortical plate. In addition, fractalkine was expressed by cells within the corpus callosum, on cell clusters within the germinal layers and in the intermediate zone, and diffusely on radiating cerebral vessels within the telencephalon. Thus, regional expression of fractalkine may also contribute to the patterns of colonisation seen during the second trimester. This is particularly significant since most recently, fractalkine produced by human astrocytes and neurons in culture, has been shown to bind to human foetal microglia and neurons which express CX_3CR1 receptors, and specifically induces microglial proliferation up to 3-fold (Hatori et al. 2002). Therefore it is possible that fractalkine expression within areas densely populated by microglial progenitors may also stimulate their proliferation *in situ*. By comparison, there was no detectable expression of SDF-1 or MIP-1β, and only minimal levels of MCP-3 or IP-10 in the cerebrum during the second trimester. The expression of chemokines within the human foetal spinal cord (9-16GW) differed from that described for the cerebrum between 16-23GW. None of the chemokines examined were found in the spinal cord during the period under examination. This may suggest that different mechanisms operate within the spinal cord and cerebrum for the recruitment of microglial progenitors, which may also reflect differences in the patterns of colonisation seen within these respective sites.

Within the human foetal cerebrum, the chemokine receptors CCR5 and CXCR4 were expressed in ventricular and subventricular zones and projections of the corpus callosum, CXCR1 expression was lacking, and CCR2, although present on microglia in the intermediate zone and subplate between 17 and 22GW, progressively demonstrated widespread expression on cellular profiles within the lower cortical plate, subplate and intermediate zone, as well as within the ventricular zone towards the end of the second trimester. The sheer numerical density of cells expressing this chemokine receptor far outweighed the number of microglia located within these regions, and whereas CXCR4 expression was found to co-localise to a large extent with microglia on double-immunolabelling, CCR2 expression became more widespread within the same regions and not solely restricted to microglia. Whereas both CCR5 and CXCR4 were specifically expressed within localised areas of the spinal cord,

particularly at 9GW, neither CCR2 nor CXCR1 were found in human foetal spinal cord samples examined between 9 and 16GW. CCR5 and CXCR4 expressed within the spinal cord at around 9GW may have been related to directing the migration of neuroglial progenitors, since few microglia were present within the ependymal zone at this age. These observations further supported the concept that different cues directed migration of microglial (and neuroglial) progenitors within the cerebrum to that seen in the spinal cord during development. The finding that CCR2 is widely expressed by a variety of cells within the developing brain may be significant from the perspective of correct migration of neuronal and (astro-)glial progenitors, as well as microglia in response to the chemotactic gradient established by MCP-1. This hypothesis is supported by findings that cultured human foetal neurons and a clonally-derived human neuronal cell line (NT2.N) are able to express CCR2, CXCR4 and that NT2.N cells can also produce MCP-1 (Coughlan et al. 2000).

There is a distinct paucity of studies looking at the expression of chemokines and their receptors *in situ* within the developing nervous system, with which to compare the above findings. Nevertheless, two other studies have looked at the developmental expression of CCR3, CCR5, CXCR3 and CXCR4 (van der Meer et al. 2001; Westmoreland et al. 2002). CCR5 and CXCR4 were found in cortical neurons of the rhesus macaque, commonly co-expressed at the cell membrane and in the cytoplasm of hippocampal CA1-4 neurons, Purkinje cells, subcortical nuclei and neocortical pyramidal neurons of layers III, V and VI (Westmoreland et al. 2002). Microglia were the predominant glial cell type that expressed CCR5 and CXCR4, and the expression of these chemokine receptors increased significantly from birth, within the first nine postnatal months. mRNA expression for CCR5 and CXCR4 however, was variable or showed a slight decrease. Such a lack of correlation between mRNA and protein levels has been found under some experimental conditions (Fields, 2001; Westmoreland et al. 2002), and may partly relate to differences in the stability of mRNA, the translation of message or to the post-translational modification of proteins. In the case of chemokine receptors, these can recycle or redistribute to the cell surface from intracellular stores (Andjekovic and Pachter, 2000). By contrast, a recent study reported limited or no expression of CCR3 and CXCR4 in the human foetal brain, while CXCR3 was found from 26GW on glial cells, endothelial cells and at term in Purkinje cells of the cerebellum (van der Meer et al. 2001). Surprisingly, these authors found that the expression of CXCR4 in their samples commenced between 3 to 4 years of infancy. This report contradicts the present findings in the human foetal brain, and that of Westmoreland and colleagues (2002) who noted in the rhesus macaque, that moderate neuronal and glial expression of CXCR4 (and CCR5) began within the second trimester, and increased with developmental age beyond birth, as detected immunohistochemically and by flow cytometry. Therefore, a discrepancy in the

timing of CXCR4 expression between these studies may be due to technical differences in the detection of this receptor, namely immunohistochemical detection on snap-frozen sections of human foetal CNS used in the present study, compared to formalin-fixed paraffin-embedded archival material that was used in the study by van der Meer and colleagues. These observations will therefore require careful further analysis.

Cultures derived from human foetal CNS between 13 and 19 weeks of gestation were also screened for chemokines and receptors previously demonstrated in situ in the developing CNS. Of all the chemokines examined, constitutive expression of MIP-1α on microglia, and variable low levels of MCP-1, MIP-1α and RANTES were found on human foetal astrocytes under basal conditions *in vitro*. In these non-stimulated culture systems, foetal astrocyte-microglial co-cultures did not express MIP-1β, MCP-3, IL-8, IP-10 or SDF-1 at the protein level as detected by immunocytochemistry. This seeming lack of expression may be attributable to a number of factors including the culture conditions, but appropriately reflects the equally apparent absence of expression of these particular chemokines in astrocytes and microglia in the normal human foetal CNS *in situ*, as shown above. By contrast, inflammatory cytokine such as TNF-α, IL-1β and IFN-γ can induce expression of IP-10, IL-8, MCP-1 and RANTES by human astrocytes (Oh et al. 1999). In the present culture system, following treatment of with LPS, only MCP-1 was significantly upregulated by glial cells. Human foetal astrocytes have been shown to express a number of chemokine receptors including CCR1, CCR3, CCR5, CXCR2 and CXCR4 (Dorf et al., 2000). Of the chemokine receptors examined, CCR2 expression was localised to clusters of astroglial precursors and less frequently in astrocytes, but could be upregulated by treatment of cultures with recombinant MCP-1. The expression of CCR2 by human foetal astrocytes maintained in tissue culture has now been confirmed by an independent group (Andjelkovic et al. 2002). CXCR4 was widely expressed by glial cells under basal conditions and was not appreciably altered by LPS treatment. CCR5 and CXCR1 could not be detected immunochemically on human foetal astrocytes or microglia in non-stimulated cultures.

Recombinant human chemokines MCP-1 and MIP-1α further acted as proliferative cues for second trimester human foetal astrocytes in a dose-response manner. This response was likely to be elicited via CCR2 receptors with MCP-1, or via the newly described CCR11 receptor (Murphy et al., 2000). The expression of CCR5 (a receptor for MIP-1α) has been noted on human foetal astrocytes (Klein et al., 1999; McManus et al., 2000b; Sabri et al., 1999), and RANTES, an alternative ligand for this receptor, promotes the proliferation of astrocyte progenitors derived from 5 and 10 week old human foetal CNS (Bakhiet et al., 2001). However, as expression of CCR5 could not be detected in non-stimulated glial cultures, it is

instead proposed that the effects of MIP-1α occur through interaction with CCR1 on astrocytes (Han et al., 2000; Tanabe et al., 1997). The possibility of 'cross-talk' between chemokine receptors however, cannot be entirely eliminated.

MIP-1α labelled microglia in subconfluent cultures and was diffusely expressed at the surface of astrocytes and at junctions between astrocytes once these cells had attained confluence. However, in contrast to other studies (Janabi et al., 1999; Miyamoto and Kim, 1999), human foetal astrocytes in these cultures did not express *consistent* levels of MIP-1α under basal conditions. Microglia, therefore represented the principal source of this chemokine *in vitro* as previously noted (McManus et al. 1998). Microglia co-cultured with astrocytes or purified and maintained *in vitro*, demonstrated expression of MIP-1α. Nevertheless, not all microglia in these isolated preparations were immunoreactive. Instead, this chemokine localised mainly to the surface and was apparent on paired cells, which implied recent division, similar to the observations recorded *in situ* in the developing CNS. By comparison, in a separate study using human foetal microglia, Peterson et al. (1997) found minimal expression of MIP-1α in the supernatant of isolated cultures maintained for 18-48 hours, as detected by ELISA (although production of this chemokine was evident when microglia were cultured in medium supplemented with LPS or recombinant IL-1β /TNF-α). It was not stated whether MIP-1α was also detected *in situ* on cells in culture. Therefore the apparent differences between the basal expression of MIP-1α by human foetal microglia can be explained by [i] different culture conditions (long-term co-culture with astrocytes and isolated preparations in the present investigation versus short-term isolated cultures), or [ii] the methods of detection of the chemokine: *in situ* on microglia as shown here versus release into the supernatant.

It is possible that co-culture with astrocytes regulates expression of MIP-1α by human foetal microglia. Furthermore basal expression of MIP-1α appears to be restricted to the surface of microglia (perhaps 'bound' to surface glycosaminoglycans) whereas further stimulation of these cells (with LPS or recombinant cytokines IL-1β or TNF-α) promotes release into the supernatant. Clarification of these issues certainly warrants further investigation. This particular chemokine is known to inhibit the proliferation of myeloid progenitors (Graham and Pragnell, 1992), is mainly produced by activated cells of myeloid or lymphoid origin including macrophages of the bone marrow (Graham et al. 1990), and is chemotactic for monocytes (Wang et al., 1993). It inhibits stem cell proliferation, but enhances committed GM-CSF and M-CSF induced colony formation (Broxmeyer et al., 1989; Graham et al., 1990; 1992; Keller et al., 1994). Murine MIP-1α enhances the myelopoietic effects of GM-CSF in culture (Broxmeyer et al. 1989), and exerts an autocrine effect on macrophage proliferation,

reported to induce TNF-α production in these cells (Fahey et al. 1992). The concentrations of MIP-1α required to elicit an inflammatory response are at least three orders of magnitude higher than that required for its inhibitory effects. It is at present unknown whether expression of MIP-1α by microglia and astrocytes regulates microglial proliferation in a similar manner to the effects observed on myeloid stem cells, and whether MIP-1α maintains these cells in a differentiated state.

Traditionally astrocytes have been regarded as a cellular source of MCP-1 (Andjelkovic et al., 2000; Barna et al., 1994; Barnes et al., 1996; Glabinski et al., 1996; Gourmala et al., 1997; Hayashi et al., 1995; Hurwitz et al., 1995; McManus et al., 2000; Weiss et al., 1998). Recently, neurons are also potential candidates for MCP-1 production (Coughlan et al., 2000; Meng et al., 1999). The expression of MCP-1 in this study was confined to clusters of astrocytes in subconfluent cultures, where this chemokine was predominantly located within intracytoplasmic vesicles. Importantly, with progressive confluence, astrocyte cultures showed MCP-1 expression at the surface and in distinct intense patches throughout cultures, which coincided with overlying, progressively ramified microglia. THP-1 monocytes were also noted to bind to isolated astrocytes that expressed MCP-1 in co-cultures, albeit not exclusively. The results presented here correspond well with the report by Andjelkovic and colleagues (2000). These authors found that while neither astrocytes nor monocytes alone produce detectable levels of MCP-1, co-culture of these two cell types led to time-dependent production of this chemokine. Specifically, the production of MCP-1 was dependent on physical contact between monocytes and astrocytes and involved the engagement of cell adhesion molecules ICAM-1 and VCAM-1, possibly mediated via release of IL-1β and TNF-α (Andjelkovic et al. 2000). In the developing human CNS, expression of this chemokine within the subplate similarly coincided with the accumulation of progressively more ramified microglia. Low levels of MCP-1 may therefore help to confine cells to distinct areas, and promote their differentiation. Although recombinant human chemokines exerted variable effects on MCP-1 expression between samples, treatment with LPS significantly upregulated expression of this chemokine by human foetal astrocytes in a reproducible manner.

MCP-1, also known as monocyte chemotactic and activating factor (MCAF), was initially purified by two independent groups based on its ability to chemoattract monocytes. Subsequent cloning and sequencing of MCP-1 showed this protein to be identical to the product of the murine PDGF-inducible JE gene (originally identified in mouse fibroblasts) (Cochran et al. 1983). Human MCP-1 cDNA encodes a 99-amino acid residue precursor protein with 23-residue hydrophobic signal peptide that is cleaved to generate a 76 residue mature protein. MCP-1 is heterogeneous in size due to addition of O-linked carbohydrates and

sialic acid residues. It can be expressed constitutively or following stimulation in a number of cells including fibroblasts, tumour cells, smooth muscle cells, endothelial cells and mononuclear phagocytes. MCP-1 appears particularly important since it has been demonstrated to specifically induce chemotaxis of microglia and recruit monocytes to the CNS (Bell et al., 1996; Hayashi et al., 1995). Furthermore, transgenic mice overexpressing MCP-1 (linked to a myelin basic protein promoter) have higher numbers of mononuclear phagocytes in the CNS (Fuentes et al., 1995). The amino terminal domain is specifically responsible for chemotaxis of monocytes (van Damme et al. 1992). This chemotactic response to MCP-1 is elicited via the CCR2 receptor G-protein (the major receptor for MCP-1), linked to cytoplasmic activation of mitogen-associated protein kinase (MAPK) (Yen et al., 1997). The existence of binding sites for β-chemokines (MCP-1, MCP-3, MIP-α and MIP-1β) on human foetal astrocytes has been suggested from binding assays (Andjelkovic et al., 1999). CCR3 (Simpson et al., 2000) and CCR5 (Klein et al., 1999; Rottman et al., 1997; Sabri et al., 1999) have previously been identified on human astrocytes. Constitutive expression of CCR2, the receptor for MCP-1 was found in human foetal astrocytes and astrocyte progenitors maintained under basal culture conditions. This appears to be associated with the state of differentiation and activity of astrocytes, since higher levels of CCR2 expression were noted on motile precursor cells and much lower levels in differentiated type 1 astrocytes. Furthermore, treatment with recombinant human MCP-1 specifically upregulated CCR2 expression in human foetal astrocytes over an 18-hour period *in vitro*. Expression of this receptor by certain populations of human foetal microglia has now been confirmed by an independent group (Andjelkovic et al. 2002). The chemotaxis of astrocytes and astroglial progenitors towards cytokines and classical chemoattractants is recognised from previous studies in culture (Armstrong et al., 1990, 1991; Bressler et al., 1985; Rowen and Glaser, 1985; Senior et al., 1986; Tanabe et al. 997). It is therefore possible that MCP-1 also acts as a functional and chemotactic signal for human foetal glial progenitors in the developing central nervous system.

The data regarding CXCR4 expression by human foetal microglia and astrocytes is in agreement with other studies (Albright et al., 1999; Gabuzda and Wang, 1999; He et al., 1997; Klein et al., 1999; Sabri et al., 1999; Shieh et al., 1998; Vallat et al., 1998). As previously noted, the pattern of 'perinuclear' intracytoplasmic immunoreactivity for both CCR2 and CXCR4 in this study can be attributed to accumulation within the Golgi apparatus, an observation previously noted in other cell types (Halks-Miller et al., 1997; Lore et al., 1998). Presumably, the intracytoplasmic expression of these receptors is a preliminary step towards expression at the surface of the cell and requires further triggering signal(s) for this purpose, present only on certain cells (including microglia and dividing astrocytes). For example, in

rodents type I astrocytes and microglia expressing CXCR4 respond to SDF-1 by mobilising intracellular calcium (Bajetto et al., 1999; Tanabe et al., 1997).

CXCR4 is constitutively expressed in a wide range of tissues including brain, heart, kidney, liver, lung and spleen (Ma et al., 1998). During early development, CXCR4 is expressed in both haematopoietic organs and proliferative areas of the brain (Jazin et al., 1997; Zou et al., 1998): migrating cells (mesoderm and definitive ectoderm) contain CXCR4 mRNA while the ligand for this receptor, SDF-1, is expressed by embryonic ectoderm (McGrath et al., 1999). Temporo-spatial interaction between this receptor and its ligand(s) appear to be critical in correct neuronal patterning of the cerebellum during development in the mouse, and deletion of the respective genes is embryologically lethal (Ma et al., 1998; Zou et al., 1998). Since ligation of CXCR4 has been shown to induce apoptosis in human neurons, it has been proposed that correct neuronal patterning results from the apoptotic elimination of cells that have migrated incorrectly (Hesselgesser et al., 1998; Horuk, 1998; Zheng et al. 1999). However, this study failed to detect expression of SDF-1, the ligand for CXCR4, either in culture in this investigation or *in situ* in the human foetal cerebrum. It is possible that additional as yet unidentified ligand(s) for CXCR4 exist within the CNS. Alternatively, it is possible that expression of this chemokine is developmentally regulated and absent or minimal during the period of development under study. Similar to the report in rodents (Ohtani et al., 1998), the levels of CXCR4 expression on human foetal glial cells were not appreciably altered by stimulation of cultures with bacterial LPS (or the recombinant human MCP-1 and MIP-1α) in this study.

To summarise, the colonisation of the CNS by microglia *in situ* necessitates long-distance migration of progenitors from specific sites to reach their target locations. Within the neocortex, this migration is brought about in part through association with radial glial elements, as well as axonal tracts. This behaviour appears similar to that of cells migrating along a gradient of chemoattractant. The temporo-spatial expression of MCP-1, MIP-1α, RANTES, IL-8 and fractalkine *in situ* in the human foetal CNS supports a role for these chemokines in directing the colonisation of microglial progenitors. A summary figure of the signals associated with the colonisation of microglial progenitors in the human foetal telencephalon is presented pictographically in **Figure 103**. The distinct expression patterns for chemokines such as MCP-1 (Meng et al. 1999; Pousset 1994), as shown in this study may also suggest additional specific roles for these factors at different stages of development, related to the cytoarchitectural organisation of the CNS. For example, related to (i) chemoattraction of glial precursors from ventricular and subventricular layers, (ii) promoting initial, random neurite outgrowth, and (iii) migration of postmitotic neurons to their specific adult locations.

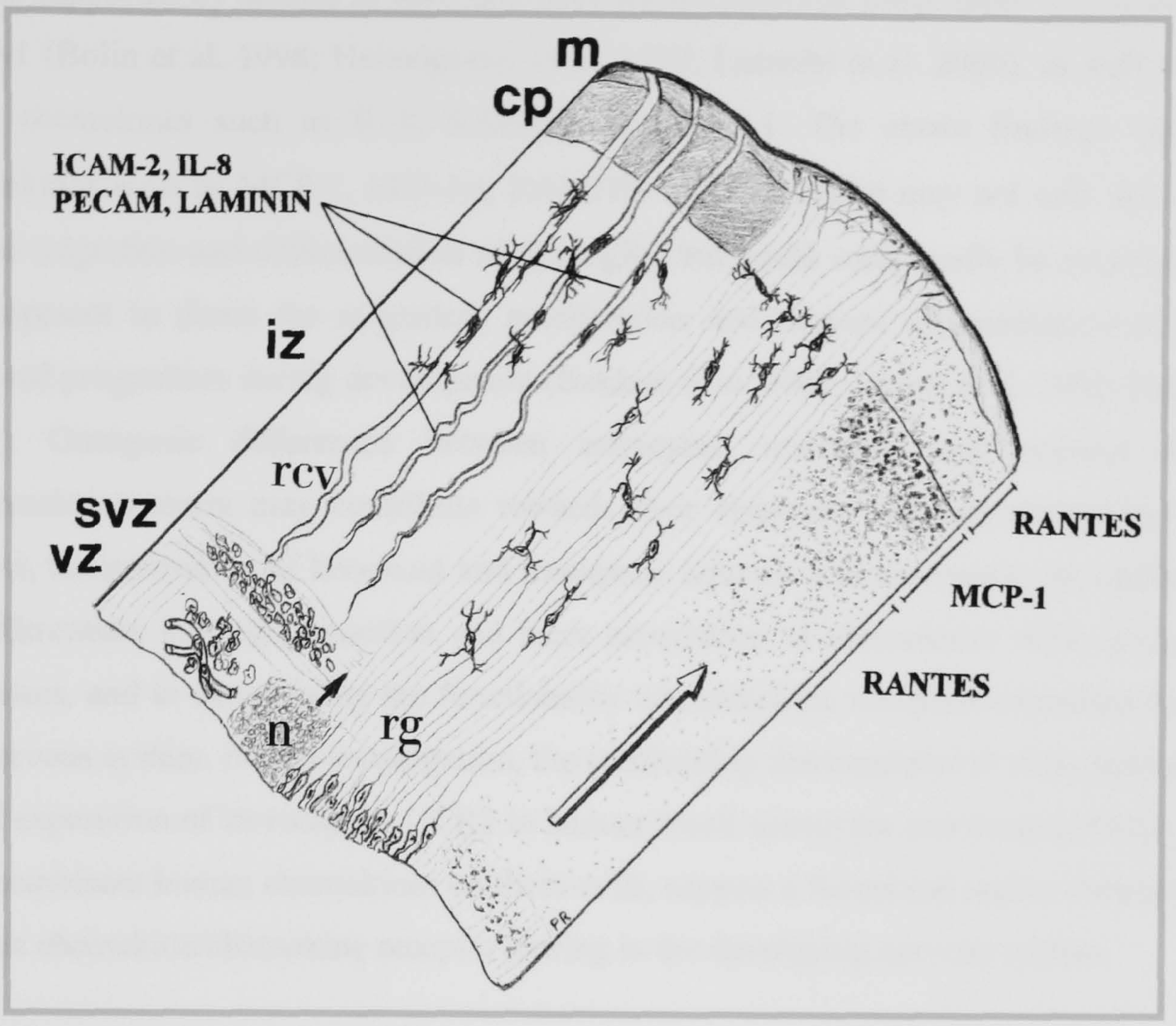


FIGURE 103

Summary diagram to show the distribution and colonisation of human cerebral cortex by microglia, 16-22GW

Microglia arising from the germinal layer associate with radiating cortical vessels and specifically with radial glial fibers as they migrate towards the cortical plate, and progressively increasing concentrations of the chemokines MCP-1 and RANTES. Foetal microglia adopt transitional morphologies in advancing through the intermediate zone. They are aligned below, but do not penetrate the cortical plate prior to 22GW.

Abbreviations: (vz) ventricular zone, (svz) subventricular zone, (iz) intermediate zone, (cp) cortical plate, (m) meninges, (rcv) radiating cortical vessel, (n) neuronal progenitors, (rg) radial glial astrocytes. Arrow indicates the direction of microglial colonisation/differentiation along chemokine gradients towards higher concentrations of RANTES and MCP-1.

This is supported by studies *in vitro* that have shown neuronal progenitors to migrate towards MCP-1 (Bolin et al. 1998; Hesselgesser et al. 1997; Lazarini et al. 2000), as well as towards other chemokines such as IL-8, RANTES and SDF-1. The above findings suggest that chemokines such as MCP-1, MIP-1α, RANTES and fractalkine may not only act as signals for the migration and differentiation of microglia, but could additionally be employed during development to direct the migration, proliferation and perhaps differentiation of glial and neuronal progenitors during development (Bakhiet et al. 2001; Hatori et al. 2002; Rezaie et al. 2002). Ontogenic differences between astrocytes, microglia and neurons and their differentiation states may contribute towards their responses towards chemokines. In this respect, the generation of knockout and transgenic animals will continue to be useful in order to differentiate between causation and mere association of chemokines with developmental responses, and in determining the functionality of chemokine receptors expressed by cells of the nervous system. In this investigation, the relationship demonstrated *in vitro* between MCP-1 and expression of its receptor, CCR2 in human foetal astrocytes and the proliferative effects of recombinant human chemokines on these cells, support a functional and/or chemotactic role for this chemokine/chemokine receptor pairing in the developing nervous system.

Colonisation of other tissues by mononuclear phagocytes during the second trimester

Does colonisation of other foetal tissues follow the same time-frame as for microglial colonisation of the CNS, and do the progenitors for resident mononuclear phagocytes use a similar array of cues to gain access to developing tissues? By comparison with the CNS, there is little information on the precise mechanisms by which mononuclear phagocytes enter other developing tissues, and these important questions remain for the most part unanswered at present. Nevertheless, studies carried out on the developing skin, another tissue also derived from embryonic ectoderm, have been promising. The epidermis contains specialised monocyte-derived dendritic cells called Langerhans cells (LCs). These function as antigen presenting cells, and can migrate from the skin via dermal lymphatics to regional lymph nodes where they present antigen to T cells and initiate primary immune responses against antigens encountered in the skin (Wang et al. 1999). To this effect, LCs first dissociate from keratinocytes, traverse the basement membrane of the dermal-epidermal junction and proceed via afferent lymphatics to draining lymph nodes. The interaction of cytokines and chemokines with appropriate receptors by LCs orchestrates their mobilisation from the epidermis and correct positioning within the paracortex of lymph nodes (Kimber et al. 2000; Wang et al. 1999). It has been proposed that bone marrow-derived, myeloid LC precursors traffic via the peripheral blood, through the dermis to the overlying epidermis (Stroebel et al. 1997).

In man, foetal LCs migrate to the skin during the first and second trimesters (Fujita et al. 1991). By 17-18GW, these cells begin to mature phenotypically (expressing CD1a and Lag antigens). Regional variations in the development of Langerhans cells have also been noted: epidermal cells of the palms of the hands and soles of the feet reach a peak in numbers in the first trimester, but are rarely detected after 18GW, whereas in other regions, their numbers increases with gestational age. Similarly, Gibran and colleagues (1996) found that dermal DCs expressing factor XIIIa (a coagulation transglutaminase) populated the dermis from about 6GW onwards and attained an adult-like distribution by 22GW. Factor XIIIa positive DCs were evident in the embryonic skin prior to the onset of bone marrow haematopoiesis and the majority expressed CD68 antigen (Gibran et al. 1996).

By comparison, in the rat primitive mononuclear phagocytes have been shown to migrate into the epidermis of the foetal rat at E17 and express an antigenic determinant (RED-1) that is present on LCs, dendritic cells, monocytes and macrophages in various organs and tissues in adult rodents (Mizoguchi et al. 1992). These RED-1 positive cells proliferate, express Ia antigen at E18, and differentiate into dendritic cells and LCs within a few days after birth. A separate group has found that foetal mouse skin is colonised by myeloid precursors that possess a macrophage/immature DC-like phenotype (H-2d,b+, I-Ad,B+, 2.4G2+, 2F8+, Sca-2+, CD11b+, CD11c+, F4/80+, B7.2+, CD44+, CD54+), and these cells can be stimulated to proliferate with GM-CSF, but not M-CSF (Girolomoni et al. 1995).

The timing of colonisation of the skin and differentiation of these epidermal monocyte-derived cells found in both man and rodents, correlate well with the period of colonisation of the CNS by microglial progenitors and their subsequent differentiation. The question that emerges is whether any similarities can be found in the expression of signals within these two 'ectoderm-derived tissues', that would preferentially recruit mononuclear phagocytes to their respective sites. Recent studies have provided exciting data particularly relating to the controlled expression of the chemokine MCP-1 in the skin. Nakamura and colleagues (1995) have shown that transgenic mice overexpressing MCP-1 in the basal layer of epidermis display a dramatic increase and redistribution of CD45+, Ia+ cells that assume dendritic morphology *in situ*, including a subset that expressed markers characteristic of LCs. MCP-1, produced mainly by keratinocytes, was specifically upregulated in the basal layer of the epidermis. Strikingly, the recruitment of these cells occurred in the absence of inflammation. Normal adult skin shows minimal levels of MCP-1, but this is upregulated in diseased skin, and increased MCP-1 expression is associated with increased numbers of CD68+ dendritic epidermal cells in various diseases of the skin (Nakamura et al. 1998). In transgenic mice, MCP-1 has been shown to specifically recruit these CD68+ cells, which represent a subpopulation of the CD1a+

Langerhans cells (Nakamura et al. 1995). Most recently, Merad and colleagues (2002) showed that resident Langerhans cells of the skin were long-lived cells with low turnover from the bone marrow. They showed that in lethally radiated mice, dendritic cells were almost completely replaced by cells of donor origin, whereas LCs of host origin remained for at least 18 months. Significantly, these authors demonstrated that under UV-light, LCs rapidly disappeared and were replaced by circulating precursors within a fortnight, and further that the recruitment of these newly-derived cells was dependent on their expression of CCR2 and on the release of chemokines targeting this receptor from the tissue. Based on these observations, when taken together with the findings presented in this work, and recent characterisations that suggest LCs are derived from monocytes, these data support a role for MCP-1 in directing progenitors of tissue-resident mononuclear phagocytes to the skin and the CNS during development. Other than MCP-1, additional factors that are involved in the specific constitutive recruitment of LCs to the skin include MIP-3α and TGF-β (Charbonnier et al. 1999; Godefroy et al. 2001; Larregina et al. 2001; Nakamura et al. 1999). The selective recruitment of LCs into the dermis can also be induced in the adult by intradermal injection of recombinant GM-CSF (Kaplan et al. 1992). However, one should still exercise caution with respect to exactly which type of cell is recruited to the skin in these studies, since normal human skin contains several different populations of cells with dendritic morphology (Nestle and Nickoloff, 1995), and the relationship between dermal dendritic cells and epidermal LCs is still unclear. It remains to be seen whether these same factors are expressed in the developing human CNS and if so, whether they are involved in the recruitment of microglial progenitors to this tissue.

Microglia in the developing mouse brain

Studies that have investigated microglia in the developing rodent brain have focused mainly around the perinatal and more intensively on the postnatal periods of development, since these are the times when an abundant, yet transient population of amoeboid cells can be identified using conventional methods, in addition to the emerging population of ramified cells (Chamak et al. 1995; DeGroot et al., 1992; Miyake et al. 1984; Perry et al. 1985; Wang et al., 1996; Wu et al., 1992, 1993). Although these periods of microglial colonisation in the mouse appear to differ from the observations in man, which occurs predominantly during the second trimester as shown in Chapter II, the timing is in fact comparable in relation to the developmental stages in humans and rodents. In humans, the onset of bone marrow formation in the humerus (embryos of 30mm crown-rump length) has been adopted by the Carnegie staging system as one of the criteria for distinguishing between the embryonic period and commencement of the foetal period of prenatal life (8 or more weeks of gestation). In the mouse, this corresponds approximately to 14-15 days of gestation (Theiler stage 23) (Theiler 1989). Further

extrapolation determines that a gestational age of 14-20 weeks in man corresponds to postnatal days 6-12 in rodents. These are periods when microglial colonisation is prevalent in both species.

It is clear from this investigation that microglial progenitors, amoeboid microglia and differentiating cells are already present at E15 in the mouse brain. Furthermore, the differentiation of microglia from amoeboid to early ramified cells is already underway during the latter half of the embryonic period, from E15 onwards. Microglial progenitors closely adherent to the parenchymal wall of cerebral vessels could be identified clearly using lectin histochemistry with *lycopersicon esculentum* and RCA-1, whereas these cells did not express markers of mononuclear phagocytes (CD11b, CD45, MOMA) and a smaller population expressed F4/80 antigen (a marker found on differentiated macrophages and monocyte-derived dendritic cells). RCA-1 in particular, clearly identified all populations of microglia in the embryonic CNS, and showed that the early ramifying cell types increase progressively in numbers. Furthermore, the period of differentiation from amoeboid to early ramified microglia was identified between E17 to E19, coincident with the progressive downregulation of binding sites for tomato lectin and F4/80 antigen on ramifying cells. This morphological differentiation also coincided with the regional maturation of cerebral blood vessels according to caudal-rostral and ventral-dorsal axes. Differences between microglia colonisation of the murine CNS from that in man may further reflect the differences in the timing and structural organisation of the nervous system (particularly related to neuronal and vascular organisation).

The topographical distribution of microglia in the murine CNS is in line with previous studies (Chamak et al. 1995; Dalmau et al. 1997; Earle and Mitrofanis, 1997; Ling 1979; Milligan et al., 1991; Miyake et al., 1984; Perry et al. 1985; Wang et al., 1996; Wu et al., 1992, 1993). For example, Perry and colleagues (1985) demonstrated early microglia/macrophages in the white matter of the developing cortex, the corpus callosum, with many cells detected within the choroid plexus and widely distributed in the pia at E16. In this study, amoeboid cells were generally more abundant in developing white matter tracts such as the corpus callosum, internal and external capsules, whereas ramifying cells were more frequently distributed additionally within grey areas (such as the caudate and thalamus), with the exception of the developing cortical plate which lacked these cells up to birth. This supports a white to grey axis for the migration and differentiation of microglial progenitors, much like that seen in the human foetal nervous system. A striking feature during murine development however, was the transient population of amoeboid cells evident between E19-E20 in the cavum septum pellucidum. Once again, this observation was in keeping with other reports (Tseng et al., 1982; Li et al., 1997).

The timing of differentiation for microglial progenitors also corresponds well with the reports by Chugani and colleagues (1991) and Alliot and co-workers (1991). Chugani et al. (1991) demonstrated that vaults (ribonucleoprotein particles) that were highly enriched in mononuclear phagocytes, were differentially expressed on microglial populations during development. They found between E15 and E18, a population of ramified cells that were ED1 and OX-42 negative, but could be detected by GSB4 isolectin and by vault immunoreactivity. Postnatally, vault immunofluorescence was also present on amoeboid cells, which expressed high levels ED1 antigen, as well as on ramified microglia, and both of these markers were downregulated by P14. These authors also noted that early ramifying cells could be detected earlier in the parenchyma than the influx of amoeboid cells detected postnatally. Thus the first component of the microglial population was made up differentiating cells between E15 and E18, and a second migration occurred in the first postnatal week, when amoeboid microglia appeared in the corpus callosum and other large fiber tracts, these cells likely to be derived from circulating monocytes. In a separate study, Alliot and co-workers (1991) found that CD11b (Mac-1) labelled cells were located in the brain parenchyma from E16 in the mouse, and the numbers of CD11b+ F4/80+ cells increased between E16 and E19. In culture, these progenitors appeared to be CD11b+ F4/80-. Based on the phenotype of these cells and their capacity to proliferate *in vitro*, giving rise to clones of CD11b+, Fc receptor+ F4/80+ cells (much like progenitors found in the bone marrow), these authors proposed that the initial progenitors of microglia could represent cells of the CFU-GM type that could give rise to granulocytes or monocytes depending on appropriate cytokine stimulation.

The finding that the differentiation of microglia coincided with the regional maturation of blood vessels in both rodents and human development, raises the question whether the same signals within the CNS drive the maturation of microglia and endothelial cells, or whether these two cell types can mutually influence each other's differentiation. Since microglia are still present in considerable numbers in mice deficient in M-CSF, the embryonic mouse brains were screened for expression of GM-CSF, to determine whether this cytokine was present within the developing brain. The results showed a lack of expression within the brain parenchyma between E15 and birth, consistent with a previous report (Chang et al. 1994). Nevertheless, the importance of GM-CSF in regulating microglial differentiation would be more suitably addressed by investigating embryonic brains from GM-CSF knockout or combined M-CSF/GM-CSF knockout mice.

Environmental factors that maintain microglia in the resting state *in situ*, also remain unclear. By comparison, a wide spectrum of factors are known to induce ramification of microglia in tissue culture, as outlined previously (**Figure 101**). However, recent evidence from studies on

CD200 knockout mice indicate that this membrane protein enriched on neurons, may exert inhibitory control on the activation and morphology of macrophages and microglia, through interaction with the corresponding CD200 receptor restricted to cells of the myeloid lineage (Barclay et al. 2002; Hoek et al. 2000; Wright et al. 2000, 2001). The expression patterns for this molecule and its receptor within the developing brain will certainly prove informative from the perspective of microglial differentiation. Brown and Perry (1998) further noted the preferential adhesion of murine macrophages and microglia to neurons in a modified *in situ* adhesion assay, on sections of the adult mouse brain. Adhesion of macrophages to neurons in the mouse may partially involve interaction with an 110Kd protein (recognised by the monoclonal antibody HB1) on macrophages (Brown et al. 1998). This same protein is recognised by the lectin GSB4 (Brown et al. 1998). It remains to be seen whether different populations of neurons could express or secrete factors (including chemokines and adhesion molecules) that attract microglial progenitors and/or influence their differentiation to ramified cells in the embryonic environment.

Thus far, we have gained a comprehensive impression of the timing, phases and potential signals recruiting microglial progenitors to the developing nervous system. Two further topics related to this work will also require examination. The first concerns functional roles for microglia during development. The second addresses the important question of the origin of these cells in the context of newly revised concepts regarding the system of mononuclear phagocytes.

Functional roles of microglia during development

Microglia have been regarded as a component of the mononuclear phagocyte system, in accord with their functional and phenotypic characteristics. From the above discussions, it is clear that selective expression of particular markers by microglia at various stages of development, may reflect functional specialisation of these cells in response to local environmental cues, particularly derived from astrocytes (Giulian et al. 1995; Sievers et al. 1994; Tanaka and Maeda, 1996). We have ascertained that microglia colonise the human foetal CNS during the second trimester, and establish their territorial 'domains' by the start of the third trimester. This period coincides with that which encompasses the greatest activity within the foetal CNS, a time when neuronal migration to the cortical plate is complete and synaptogenesis and neuronal differentiation have commenced (Chan et al. 2002). It is reasonable to assume therefore, that the infiltration of microglia within this period of heightened structural organisation serves a fundamental purpose. The exact nature of this purpose however, remains speculative at present. Nevertheless, it is necessary to evaluate the potential contribution of microglia towards CNS histogenesis and tissue modelling during

development. In order to place such hypotheses into context, mention must first be made of the developmental processes that abound during the second trimester.

The remarkably complex organisation of the cerebrum follows a series of highly co-ordinated temporally and spatially-regulated sequence of events, which are heightened during the second trimester. These involve the generation, migration and maturation of neurons destined for the cortical plate and glial cells within germinal layers, as we have already seen (Choi, 1988). During this period, post-mitotic neurons migrate in highly restricted fields along fibers of radial glia, which span the thickness of the telencephalon extending from the ependyma to the leptomeninges (Choi, 1988; Gould and Howard, 1987; Hatten, 1990; Rakic 1981; Sasaki et al. 1988; Wilkinson et al. 1990). The marginal zone (Layer I) develops early on, following the establishment of the pia-glial barrier. Migration and maturation of Cajal-Retzius neurons to this layer appears critical in determining the subsequent growth and layerwise arrangement of the cortical plate (Chan et al. 2002). Cortical plate neurons that arrive first, are destined for deeper layers, and successive waves of neurons arriving at the same location, populate more superficial layers in an inside-out fashion (Choi, 1988). This developmental process raises two important points for discussion. The first concerns radial glial tracts that are established for the migration of neuronal progenitors and glial precursors to the developing cortical plate. There is undoubtedly a close association between foetal microglia and vimentin+/GFAP+ radial glia and astrocytes both *in situ* and in tissue culture, as shown in this investigation, to the extent that microglia are absent from areas devoid of radial glial projections within the intermediate zone *in situ*, and migrate on astrocytes *in vitro*. By reason of this relationship it is possible that microglia use the same structural highways (although not exclusively) as neuronal/glial progenitors in colonising the CNS.

The second matter involves signals used for triggering migration. The specific distribution of chemokines MCP-1, RANTES and fractalkine in the second trimester not only identifies signals triggering the migration, proliferation and perhaps differentiation of foetal microglia but may also be responsible for signalling specific neuronal and glial migration, neuronal patterning and neuronal-microglial interaction, as discussed previously. Evidence supporting these last hypotheses has only recently emerged. Primary sensory neurons have been shown to migrate in response to RANTES expressed *in situ*, in embryonic mice (Bolin et al. 1998). CXCR2 is widely expressed in the human CNS, particularly on subsets of projecting neurons in the hippocampus, dentate nuclei, pontine nuclei, locus coeruleus, paraventricular nucleus, as well as in the anterior horn, antero-mediolateral cell column, and Clarke's column of the spinal cord. Fibres expressing CXCR2 are located in the superior cerebellar peduncle, substantia gelatinosa and other sites of the adult nervous system (Hesselgesser et al. 1997;

Horuk et al. 1997). The importance of chemokines in the developing CNS is further realised since transgenic mice deficient in CXCR4 or SDF-1 die perinatally with severely compromised immune development, and abnormal cerebellar organisation (Ma et al. 1998). CCR3, CCR5 and CXCR4 have been detected in the human brain in a variety of cell types including microglia, astrocytes, neurons and vascular endothelium (Lavi et al. 1997; Rottman et al. 1997; Vallat et al. 1998). This study has shown that the expression of CCR2 in the human foetal CNS is not exclusive to microglia. In culture, astrocytes and their progenitors also express this chemokine receptor. Moreover, astrocytes have been shown to migrate towards certain chemokines, including MIP-1α (Tanabe et al. 1997). A human neuronal cell line which expresses CCR1, CCR5, CXCR2 and CXCR4 receptors, responds to α- and β-chemokines by migrating in the differentiated (but not undifferentiated) state (Hesselgesser et al. 1997). The idea of signalling between neurons and microglia is supported by Nishiyori et al. (1998) who demonstrated the expression of fractalkine mRNA in rat neurons, and dominant expression of its CX_3CR1 receptor mRNA in microglia *in vitro*, but not on astrocytes or neurons. Human foetal astrocytes and neurons likewise produce fractalkine in culture, the receptor for which is present on human foetal microglia (and on neurons themselves) (Hatori et al. 2002). A separate report by Hesselgesser and colleagues (1998) highlighted a different role for chemokines within the nervous system, namely the induction of neuronal apoptosis by SDF-1 acting via CXCR4. This is intriguing since this study has shown a pattern of CXCR4 expression within the germinal layers of the human cerebrum and spinal cord, which coincided with TUNEL-detection of apoptosis.

Programmed cell death is an important feature of the developing nervous system, and macrophages can be found engulfing pyknotic nuclei (Ashwell, 1991; Hume et al. 1983). Naturally occurring cell death or 'apoptosis' associated with the development of many organs, may act as a contributing stimulus for recruitment of macrophage populations (Ferrer et al. 1990; Raff et al. 1993). However, the relationship between microglial distribution and apoptotic events in the developing nervous system remains controversial. There have been reports associating the distribution of microglial progenitors with cell death (Perry et al. 1985; Perry and Gordon, 1988; Rakic and Zecevic, 2000). Macrophages have also been noted at various sites in developing tissues before neuronal death is prominent, and are not necessarily associated with foci of cell death (Ashwell 1990,1991; Diaz-Araya et al. 1995; Gould and Howard, 1991; Milligan et al. 1991; Upender and Naegele, 1999; Sorokin et al. 1994). The fact that microglia are not exclusively associated with pyknotic cells, implies that dying neurons are not the sole determinants of the distribution of these cells, but may play an important role (Perry et al. 1985). Using TUNEL labelling to identify the extent of apoptosis and CD68 immunohistochemistry on the developing cortex, this study has also noted that

although microglia appear to be associated with dying cells in the ventricular and subventricular zones, they are equally present in neighbouring areas that lack dead cells. Foetal microglia may therefore regulate earlier (or later) events. in addition to their function in removing cellular debris (Davis et al. 1994). Nevertheless, the process of colonisation of the CNS by microglia proceeds in a cohesive and orchestrated manner, which hints primarily of a development-regulated response (i.e. to a specific set of temporo-spatial cues or signalling factors). Their topographical distribution patterns correspond to specific stages in the structural organisation of the CNS (i.e. they predominate in white matter and invade grey matter only when these areas are at a more advanced stage of development structurally and functionally). Their colonisation during the mid-to-late foetal period coincides with neuronal differentiation, synaptogenesis and the development of axonal-target connections (Chan et al. 2002). The early emergence of microglial progenitors within the subplate (and intermediate zone) of the telencephalon may be important from the viewpoint that this region is known to harbour thalamocortical projections with a high degree of synaptogenesis (Chan et al. 2002), in addition to possessing the highest index of apoptosis in the developing CNS (Rakic and Zecevic, 2000).

Like their macrophage counterparts in other tissue compartments, microglia have demonstrated the potential to produce factors (bFGF, TGFβ, IL-1β, IL-8) that are associated with angiogenesis (the formation of new capillaries from pre-existing blood vessels) and tissue repair (Aldskogius, 2001; Giulian et al. 1988). Studies on neovascularisation of allografts (Pennell and Streit, 1997) and in the human foetal retina between 15 and 22GW (Provis et al. 1997) have shown microglia to be positioned in advance of ingrowing blood vessels, as well as intimately associated with vascular endothelium and vascular sprouts. At all stages of development examined in this study, microglia were noted to closely associate with vessels. During development, the formation of new blood vessels from angioblasts is referred to as vasculogenesis. Histological examination of tissue sections with ongoing angiogenesis (for example in tumours), emphasise the importance of granulocytes and macrophages as prerequisite for neovascularisation. Experimental depletion of either cell type suppresses the formation of new vessels (although the angiogenic capacity of granulocytes is inferior to that of macrophages). Macrophages release several proteases (including plasminogen activator and elastase) that may yield angiogenic fragments from ECM molecules. Several soluble factors are stored in the ECM through binding to heparin-like glycosaminoglycans (e.g. bFGF, TGF-β, GM-CSF). Enzymatic release from these 'reservoirs' would render them available to endothelial cells. For instance, macrophages can degrade heparan sulphate and release ECM-bound bFGF through expression of urokinase-type plasminogen activator. Among the cytokines produced by macrophages, TGF-β modulates the expression of fibronectin or Type I

collagen and incorporation of these molecules into the ECM, angiotropin, PDGF. and IL-6 exert modulatory roles in angiogenesis, bFGF which has been shown to stimulate the directed migration and proliferation of cultured endothelial cells and promotes the formation of differentiated capillary tubes *in vitro*, and human angiogenic factor initiates neovascularisation by inducing migration of endothelial cells to form sprouts without proliferating. Macrophages also control the extent of development of new vascular networks and promote their differentiation of vascular sprouts into functionally-mature capillaries through the release of several factors that inhibit mitosis or migration of endothelial cells: monocyte-derived endothelial cell inhibitory factor (MECIF), macrophage-derived endothelial cell inhibitor (MD-ECI), thrombospondin-1, and IFN-α. These factors promote the differentiating effects of otherwise chemotactic and mitogenic factors such as angiotropin. bFGF, GM-CSF or M-CSF. The accumulation of microglia within the CNS at sites where considerable vascular reorganisation, collateral sprouting and maturation is prevalent (for example within the germinal layers, corpus callosum, and subplate) may therefore indicate their involvement in modelling of the cerebral vasculature. In order to examine this further, one could look at the direct interaction between human foetal microglia and endothelial cells *in vitro*, and check expression of certain of these angiogenic-associated elements (including αVβ3/CD51/CD61, angiogenic cytokines such as VEGF and FGF) in cerebrovascular development in relation to microglia. The maturation of blood vessels may be followed by investigating expression of specific molecules including laminin, which is constitutively expressed on developing vessels, fibronectin, which is a developmental marker and factor VIII, a marker for the progressive maturation of blood vessels.

The reasons for the preferential location of amoeboid microglia within the white matter of the developing nervous system are certainly intriguing. These observations beg such questions as whether microglia in these localities are simply phagocytes eliminating debris and aberrant projecting axons as suggested, or whether they exert a more specific role in directing the growth and differentiation of myelinating oligodendrocytes, astrocyte proliferation and differentiation, or indeed the spatial regulation and modelling of axonal tracts. It is equally possible that amoeboid microglia are directed through the CNS by signals that accompany the formation of these tracts. As has been emphasised, amoeboid microglia that are abundant within the developing brain, closely resemble macrophages in terms of morphology. phenotype and function. In the rodent CNS, these cells are believed to phagocytose debris. which occurs as a result of naturally-occurring cell death in the late embryonic and early postnatal stages of development (Ferrer et al. 1990). and to eliminate certain axonal projections and synapses as part of the normal structural organisation of the CNS (Hume et al. 1983; Innocenti et al. 1983; Perry et al. 1985). Innocenti and co-workers (1983) in particular.

noted the capacity of amoeboid microglia to selectively eliminate non-connected cortico-cortical axons without affecting neighbouring fibers and cells. With progressive transformation to adult forms, this phagocytic capacity gradually subsides. Amoeboid cells isolated *in vitro* from the rodent CNS have been shown to secrete a variety of cytokines (including bFGF, NGF, IL-3) that may participate in gliogenesis, regulate neuronal migration and differentiation, direct axonal projections/sprouting and synaptogenesis, and even regulate myelin synthesis (Hamilton and Rome, 1994). Amoeboid microglia can also be stimulated to produce a variety of inflammatory mediators including TNF-α, IL-1β and IL-6, chemokines and free radicals, all of which are potentially damaging to the vulnerable cellular environment. However, it should be stressed that events occurring during normal development are disparate from those associated with an inflammatory response that is initiated in the adult nervous system. It is more likely that these factors, if produced in the developing CNS, are strictly controlled and exert specific effects at physiological levels. **Table 16** lists the main factors known to be expressed by or released from human foetal microglia.

Tissue culture studies indicate that microglia not only produce factors that may in essence promote neuronal survival, regeneration and plasticity, but may also influence the differentiation of particular subsets of neurons (for example GABAergic or cholinergic neurons (Mazzoni and Kenigsberg, 1997), mesencephalic neurons (Nagata et al. 1993), and promote neurite outgrowth (Nakajima et al. 1993)). Moreover, there is indirect *in vivo* evidence of a physical role for microglia in neuronal regeneration through the process of 'synaptic stripping' or 'pruning' of presynaptic terminals that abut onto a traumatised neuron. This, combined with astrocytic aid, affords the afflicted neuron the opportunity to recuperate and regenerate (Aldskogius, 2001). Experiments *in vitro* have shown that microglia-conditioned medium is neurotrophic to primary neurons, and significantly enhances the survival of cortical and mesencephalic neurons and their neurite extensions in rodents (Nagata et al. 1993). Microglia-conditioned medium also elevates dopamine uptake by dopaminergic neurons (Nakajima and Kohsaka, 1993). NGF, known to be synthesised by microglia, does not mediate this effect. It is possible that IL-1 (Giulian et al. 1986; Yao et al. 1992), IL-3, bFGF (Araujo and Cotman, 1992; Presta et al. 1995; Shimojo et al. 1991), and plasminogen also produced by microglia, may serve as neurotrophic factors promoting the survival of neurons. Platelet activating factor (PAF) produced by human foetal microglia (Jaranowska et al. 1995) has a putative role in neuronal differentiation, and signal transduction, since binding sites to PAF have been localised to synaptosomes and specific brain areas. In addition to its recognised roles in stimulating neurite outgrowth and exerting trophic influence on neurons in culture and *in situ*, bFGF is also a proliferative factor for astrocytes, oligodendrocytes, and a differentiation and proliferation factor promoting phagocytosis in microglia (DiPucchio et al.

1996). Human recombinant bFGF can induce proliferation of glioblasts in human foetal brain cultures. Microglial bFGF could in theory, prevent naturally-occurring neuronal death or exert a mitogenic effect on neuroblasts and glioblasts, since bFGF receptors are also present on immature neurons and glia in rodents and man (Presta et al. 1995).

These observations indicate a physiological significance for microglia in regulating neuronal growth, function and regeneration. Further evidence supporting a role for microglia and macrophages in promoting neurite outgrowth (collateral sprouting) is obtained from grafting studies in the *peripheral nervous system*, where these cells can promote the regeneration of sensory axons (Lazar et al. 1999; Prewitt et al. 1997; Rabchevsky and Streit, 1997). Although blood-derived macrophages have been shown to modify the properties of adult mammalian CNS white matter near mechanical lesions, and convert a non-permissive state to promoting axonal growth (David et al. 1990), to date the majority of studies addressing the *central nervous system*, do not favour a supportive function for these cells under traumatic conditions in the adult (Aldskogius, 2001). So, it still remains to be seen whether microglia could indeed exert effects favouring neurite outgrowth and neuronal differentiation *in situ* within the developing nervous system.

So far, the heterogeneity inherent in the morphology and functional biochemistry of microglia has been the cause for conflicting views on the roles of these cells during development. The source (rodent versus human tissues) and processing of material (isolated cell preparations, organotypic cultures, or paraffin-embedded versus frozen tissue sections) have additionally presented as confounding variables. Despite this, the functions currently proposed for microglia in the developing brain, are generally in keeping with their topographical distribution, phenotype and behaviours determined *in situ,* and in culture experiments (Streit, 2001): cells located preferentially within white matter tracts may carry out functional roles related to phagocytosis of aberrant axons, or they may adopt additional roles, for example in tissue and vascular remodelling, or promoting the growth of axons and neurites (for example, through the production of extracellular matrix components such as thrombospondin and growth factors including NGF and bFGF). Overall the findings highlighted above suggest a supportive role for microglia in histogenesis and modelling of the CNS during development.

Which are the progenitor cells for microglia?

In attempting to answer the question regarding the origin and nature of microglia, we first have to consider the differentiation of myeloid cells, and their respective phenotypic and functional characteristics. In the first instance, we are confronted with the necessity to distinguish events that occur during haematopoiesis in development, from that which

predominates in the adult. First, we shall examine the ontogeny and development of mononuclear phagocytes and dendritic cells. According to the classical hypothesis of the mononuclear phagocyte system, macrophages are the progeny of bone marrow-derived monocytes (van Furth et al. 1972). Mononuclear phagocytes are widely distributed throughout the body and include precursors in haematopoietic tissues, peripheral blood monocytes and free and fixed macrophages in tissues, organs and cavities. Stem cells give rise to committed progenitors along specialised cell lineages. Among these, myeloid progenitors differentiate into precursor cells including granulocyte-macrophage forming cells. The bipotential granulocyte-macrophage progenitor [CFU-GM] further differentiates to granulocytes [CFU-G] and macrophage-forming [CFU-M] cells. The monoblast (or immature macrophage) differentiates to form promonocytes, the immediate precursor of monocytes. The latter circulate in the blood and penetrate various tissues via blood vessels, where they differentiate into resident macrophages. Haematopoiesis is controlled by a system of more than eleven growth factors, and at least three glycoproteins initiate the differentiation of macrophages from uni- and bi-potential progenitor cells. The progression from a pluripotent stem cell to a myeloid-restricted progenitor is driven by IL-3 (a cytokine that generates differentiated progeny of all myeloid lineages). With differentiation, IL-3 responsive progenitors, become responsive towards M-CSF and GM-CSF (and to some extent TNF-α), the principal growth factors giving rise to monocyte/macrophage-restricted cell lineages. It is well known that M-CSF, GM-CSF and stem cell factor promote the differentiation of macrophages precursors into mature macrophages, and are important for their growth and survival (Sorokin et al. 1992, 1996). Specifically, in the past, the growth factor M-CSF was considered to specifically promote differentiation of myeloid progenitors to form macrophages. Following lineage commitment, the cells are dependent on these factors for proliferation and viability. Ultrastructurally, M-CSF-treated macrophages retain a mature appearance whereas GM-CSF-treated cells become less differentiated. Haematopoietic differentiation is therefore supported by the environmental milieu within specific organs. For example foetal rat lungs have high levels of GM-CSF, M-CSF and IL-1β (but not TNF-α) supportive of macrophage differentiation early in development (Sorokin et al. 1997). The phenotypic profile of rodent and human myeloid cells and the markers of differentiation of mononuclear phagocytes are presented in **Figure 104** for reference.

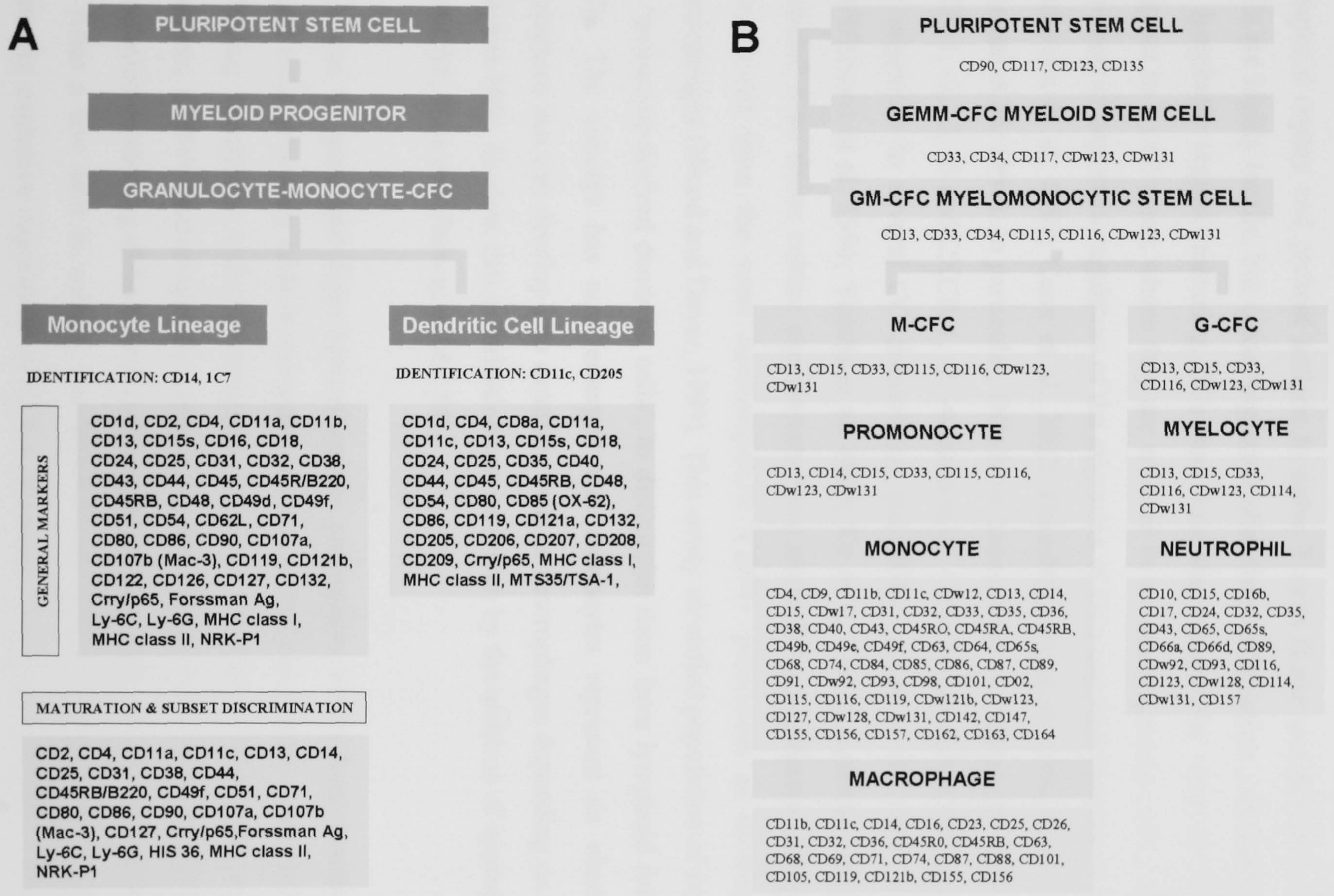
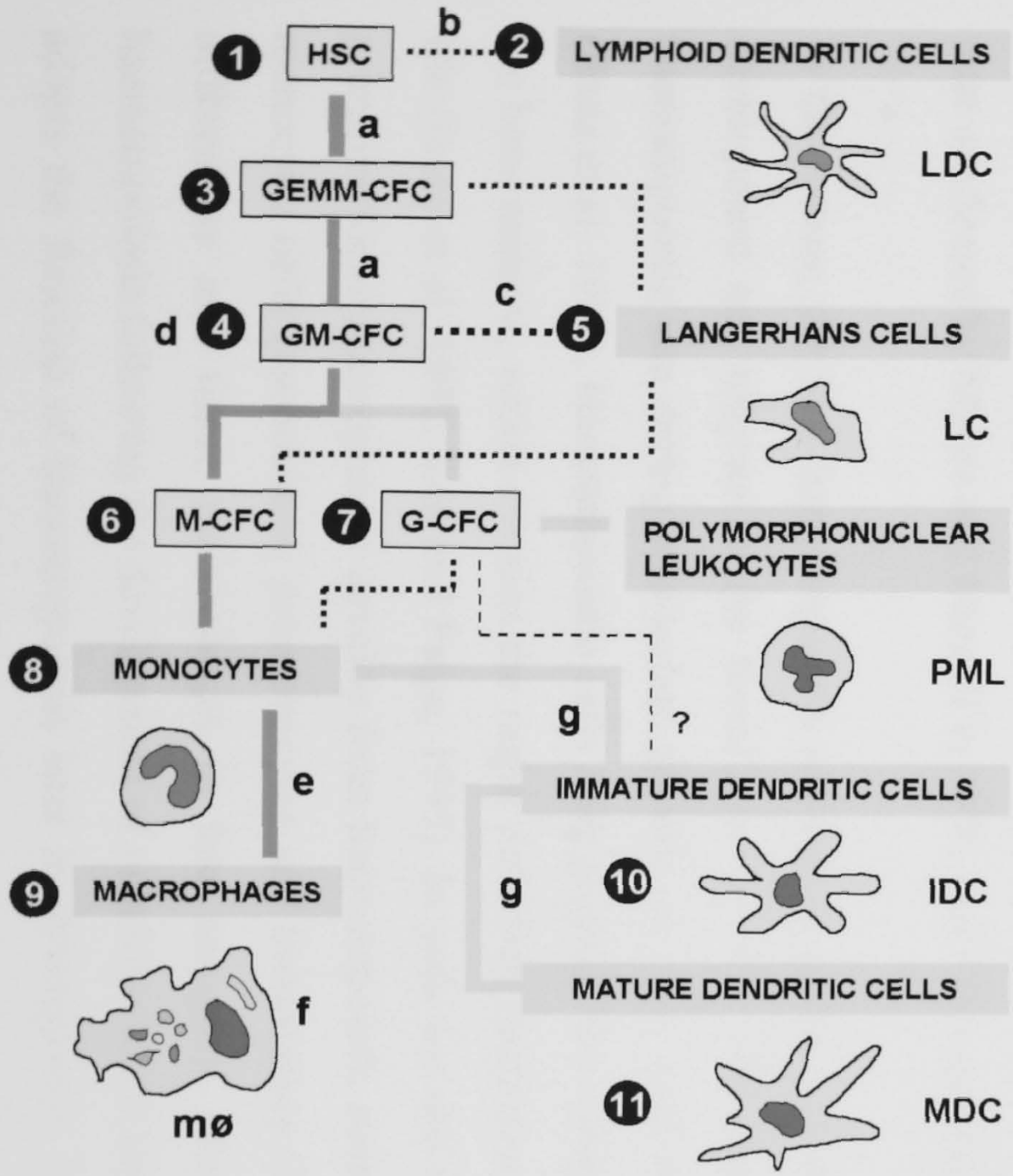


FIGURE 104

Phenotypic profile of rodent and human myeloid cells

Dendritic cells (DCs) on the other hand, have been considered for many years as representing a lineage distinct from monocytes and macrophages (Steinman, 1991). These cells form a network of highly efficient antigen-presenting cells whose function is predominantly concerned with surveillance of tissues for pathogens. Their 'sentinel' functions are primed through exposure to immune or inflammatory stimuli. Following the capture of antigen in peripheral tissues, DCs undergo a process of 'maturation' that enables them to migrate to lymphoid organs and present antigen to naïve T cells (Lane and Brocker, 1999). Immature DCs are highly motile, but cease migration after they differentiate. After having gained entry to lymphatic organs, maturing DCs pass into the subcapsular sinus of lymph nodes and migrate to T cell areas, where they are referred to as 'interdigitating cells'. Chemokines have emerged as important regulators of the migration of DCs and their homing to lymphoid organs (Allavena et al. 1999; Caux et al. 2002; Dieu-Nosjean et al. 1999, Rescigno et al. 1999, Sozzani et al. 1999). In particular, DCs have been shown to express the chemokine receptors CCR-1, -2, -3, -5 and CXCR-1, -2, -4 (Sato et al. 1999; Sozzani et al. 1997), and can respond by migration to several CC chemokines and SDF-1 (Dieu et al. 1998; Lin et al., 1998; McWilliams et al. 1996; Vecchi et al. 1999; Xu et al. 1996). Recently, experiments mainly gathered from tissue culture experiments have identified a population of dendritic cells that are derived from the same haematopoietic CD34+ precursors as monocytes and tissue macrophages (Nicod and Dayer, 1999). This newly identified population of cells is referred to as 'monocyte-derived dendritic cells', to distinguish them from lymphoid follicular dendritic cells. The concept has now emerged that monocytes represent an abundant source of precursors that can develop into dendritic cells or macrophages depending on external stimuli (**Figure 105**). *In vitro*, this polarisation can be driven by the addition of appropriate cytokines: GM-CSF, IL-4 and TNF-α, or M-CSF.

DCs can be generated from haematopoietic progenitors in the bone marrow, CD34+ cord blood cells, precursor cells in the peripheral blood and blood monocytes through appropriate culturing conditions with combinations of these cytokines (Hashimoto et al. 1999). For example, CD34+ haematopoietic progenitors from human cord blood generate a mixed cell population containing different types of dendritic cells when cultured with GM-CSF and TNF-α. After a few days in culture, two types of dendritic cell precursors can be identified by mutually exclusive expression of CD1a (a marker found on dendritic cells and Langerhans cells) and CD14 antigens. Within 1-2 weeks, CD1a precursors generate cells that express Birbeck granules (cytoplasmsic organlles), lymphocyte activation gene antigen, and E-cadherin- markers characteristic of epidermal Langerhans cells. In contrast, CD14+ precursors mature into CD14+/CD1a- dendritic cells which express CD2, CD9, CD68 and coagulation factor XIIIA found in dermal dendritic cells.



1 CD4, CD31, CD34, CD90, CD117, CD123, CD127, CD135, Fa II-3, Ly-6A/E (Sca-1), Lin

2 CD1d, CD4, CD8α, CD11b, CD11c, CD13, CD117, Ly-6A/E, MHC class II, DEC-205

3 CD31, CD34, CD90, CD117, ER-MP58, Ly-6A/E

4 CD11b, CD31, CD34, CD90, CD115, CD117, ER-MP58, Ly-6A/E, Ly-6G

5 CD11b, CD115, DEC-205, E-cadherin, ER-HR3, MHC class II, F4/80

6 CD11b, CD31, CD115, CD117, ER-MP58, Ly-6C, Ly-6G

7 CD11b, CD31, CD117, ER-MP58, Ly-6G

8 CD11b, CD115, ER-MP58, Ly-6C, Ly-6G, BM8, ER-HR3, F4/80

GENERAL

9 CD11b, CD45, CD64, CD68, CD115, F4/80, BM8, MHC class II, Scavenger R.

SUBSETS

9 CD11b, CD45, CD64, CD68, CD115, F4/80, BM8, Scavenger R.

10 CD11b, CD11c, CD13, CD68, F4/80, MHC class II

11 CD11c, CD13, CD68, MHC class II, DEC-205

PHENOTYPIC CELL DIFFERENTIATION MARKERS OF RODENT MONONUCLEAR PHAGOCYTE LINEAGE

FIGURE 105

Phenotypic markers of mononuclear phagocyte differentiation

a: Committed to myeloid lineage, **b:** thymic dendritic cells (DCs) and a subset of peripheral lymphoid DCs originate from a precursor with restricted lymphoid/NK/DC potential, **c:** in man, this pathway is distinct from monocyte-derived DCs and lymphoid DCs, but the branching point is unclear, **d:** these are the most mature common progenitors of mononuclear phagocytes, although there is some indication that cells beyond the G-CFC stage of neutrophil development may still mature into macrophages or DCs, **e:** monocytes develop into tissue macrophages. It is likely that many populations of tissue macrophages are seeded during ontogeny and are maintained by local proliferation of mature macrohages and/or their precursors, **f:** heterogeneous morphologies, functional properties and cell surface phenotype reflect tissue localisation and specialisation, **g:** the development of DCs appears to be complex and involves multiple pathways. These may also be present in macrophage development and contribute to the heterogeneity of these two cell types.

These same CD14+ precursors (but not the CD1a precursors) are able to differentiate into macrophages in response to M-CSF. Dendritic cells can also develop from CD14+ peripheral blood monocytes cultured with GM-CSF and IL-4. Under these conditions, monocytes develop into a homogeneous population of immature dendritic cells, in which maturation can be further induced through exposure to inflammatory stimuli such as TNF-α, IL-1, bacterial LPS, or by monocyte-conditioned medium and IL-13. Immature dendritic cells generated from monocytes retain expression of the M-CSF receptor, but lose it after maturation. Therefore it is becoming increasingly more evident that multiple cell types with distinct developmental pathways and functional properties constitute the system of DCs.

Mononuclear phagocytes arise from haematopoietic stem cells during embryonic, foetal and adult life. It has been thought for some time, that macrophages are the exclusive progeny of bone-marrow-derived monocytes. However, there is now accumulating evidence for differences in ontogeny between foetal and adult macrophages (Shepard and Zon, 2000). Embryonic or foetal macrophages in particular, do not necessarily follow the monocytic pathway. Studies in a variety of species indicate that foetal macrophages instead differentiate from yolk sac and hepatic-derived primitive macrophage precursors prior to the onset of development of 'adult' monocytes. As far back as 1908, Dantschakoff noted the existence of macrophages in the yolk sac prior to the initiation of bone marrow haematopoiesis. This was later confirmed by Moore and Metcalf in 1970, and Cline and Moore in 1972.

In the mouse, the first haematopoietic cells arise in the yolk sac around day 7.5 of mouse development and migrate to the foetal liver by E11. The foetal liver remains the major haematopoietic site during mid-to-late gestational ages, until about the first postnatal week (Ikuta et al. 1992). Haematopoietic cells later populate the spleen after day 15 and eventually the bone marrow, which remains the major definitive haematopoietic organ throughout adult life (Ikuta et al. 1992; Kee and Paige, 1995). In yolk sac and early hepatic haematopoiesis, primitive/foetal macrophages develop from haematopoietic stem cells bypassing the stage of monocytic cells (monoblasts, promonocytes and monocytes) (Takahashi et al. 1996). They differentiate into tissue macrophages in late ontogeny. Monocytes develop in hepatic haematopoiesis following the development of primitive/foetal macrophages. The bone marrow adopts the function of haematopoiesis later in development. In adult life, the macrophage precursors (immature myeloid cells) and monocytes are released from the bone marrow into peripheral blood. These precursors also migrate into tissues and differentiate into resident macrophages or related cells in response to differentiation and growth factors such as M-CSF and GM-CSF. By comparison, in man the first formation of blood cells takes place in blood islands in the wall of the yolk sac, and after the 6th week, blood formation takes place chiefly

in the liver, but also in the spleen and other tissues (including connective tissues). Late in the third month haematopoiesis commences in the bone marrow, and progressively, these other sites become effete.

In rodents, macrophages first appear in the blood island of the yolk sac on the ninth day and mature into foetal macrophages by day 10 (Takahashi et al. 1989; Takahashi and Naito, 1993). They leave the blood island and enter mesenchymal layer and extra-embryonic coelom before colonising various tissues via blood vessels, where they are retained as resident macrophages (Takahashi et al. 1996). These foetal macrophages possess a high proliferative capacity, typically lacking in adult macrophages. They display phagocytic capability and can be identified by immunocytochemistry to F4/80 antigen. Foetal macrophages arise from primitive macrophages prior to development of monocytes. Data from studies carried out in vertebrates and invertebrate species supports the existence of these two separate macrophage lineages: foetal and adult/monocytic (Shepard and Zon, 2000 for references). In avian species, yolk sac chimaera studies have shown macrophage-like cells of yolk sac origin with phagocytic capacity, particularly in the developing CNS associated with areas of cell death. In the zebrafish macrophages originate from the lateral mesoderm located at the anterior end of the embryo. Primitive macrophage precursors first appear in the yolk sac and migrate to the mesenchyme of the head and circulating blood. These are present before the appearance of other leukocytes, and display proliferative potential and capacity for phagocytosis. In Xenopus, creation of head-body chimaeras demonstrates that early macrophages originate from mesenchyme of the head portion of embryos. Similarly, in insects macrophages (haemocytes) derived from mesoderm of the head migrate throughout the embryonic larvae where they develop into phagocytic macrophages. In rodents, primitive macrophages can be identified in the connective tissue surrounding the brain at E12, prior to vascularisation of this organ (Sorokin et al. 1992). The predominant functions of these primitive macrophages are thought to concern removal of apoptotic cells during embryogenesis and development (Hopkinson-Woolley et al. 1994) and modelling of tissues. From these studies, it appears that foetal macrophages originate from a separate lineage of cells than monocyte-derived macrophages, and that various tissues are colonised by two 'waves' of macrophages: yolk-sac/mesenchyme-derived population of foetal macrophages that initially surround organs and later in development by blood-borne monocyte-derived mononuclear phagocytes. This distinction may partly reflect different functional requirements of foetal macrophages (such as tissue modelling) during early embryogenesis. There are also differences in the cytokine responsiveness of progenitor cells isolated from foetal liver, cord blood and adult bone marrow (Weekx et al. 1998). A summary of the proposed origins for myeloid-derived mononuclear phagocytes in the mouse is shown in **Figure 106**.

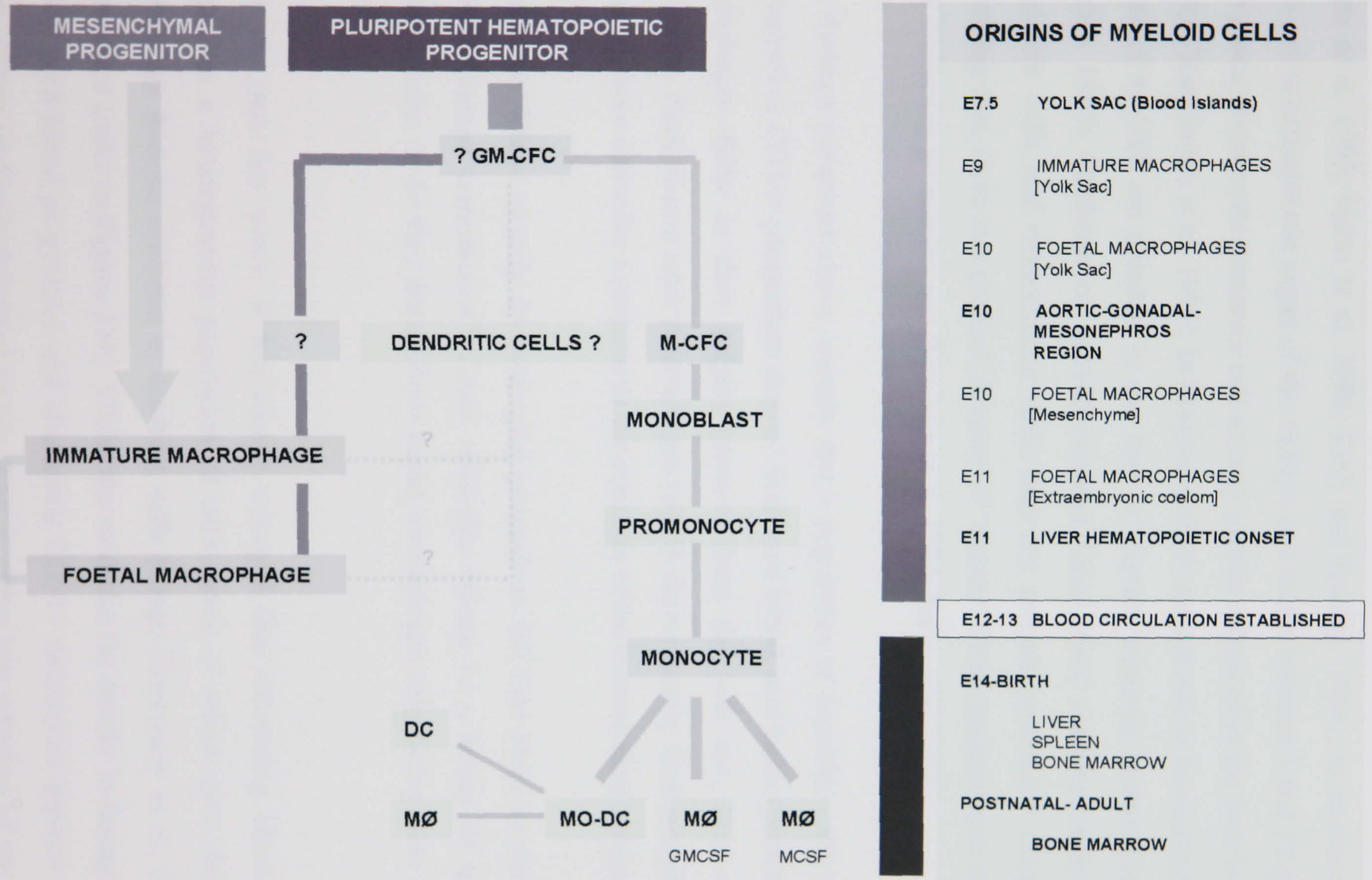


FIGURE 106

Proposed origins of myeloid-derived mononuclear phagocytes in the mouse

In man, the first cells with ultrastructural characteristics of macrophages appear in the yolk sac and mesenchyme in the fourth week of gestation, and in pre-haematopoietic liver by the fifth week. Muramidase and Mac-387 positive cells have been detected in human foetal liver as early as 7 weeks, mainly in connective tissue and occasionally within hepatic sinusoids (Bardadin et al. 1991). Consistent with the knowledge in rodents. that the liver and spleen are the major sites of haematopoiesis during early foetal development (Morioka et al.. 1994: Moris et al. 1991; Naito et al. 1990; Roth and Stanley, 1995), in humans, the liver is the principal haematopoietic organ of the embryo and foetus between 5 and 22 gestational weeks, with granulomonocytic precursor cell activity greatly enhanced in the liver between 16 and 22 weeks (Torubarova et al. 1995). In keeping with its central role in haematopoiesis, the highest levels of M-CSF are detected in the liver of second trimester human foetuses (Roth and Stanley, 1995). Furthermore, second trimester foetal blood contains much higher levels of progenitor cells and mononuclear cells than are present postnatally (Jones et al. 1994). Following this, there is a gradual increase of 'mature' foetal macrophages in various tissues, with increasing gestational age (Morioka et al. 1994; Naito et al. 1996).

The findings presented above, namely that a population of dendritic cells are derived from monocytes or CD34+ progenitors derived from cord blood, and foetal (primitive or immature) macrophages differ in their origins (derived from the yolk sac, liver and spleen) and phenotype, from mature adult macrophages (which derive mainly from progenitors in the bone marrow), have called for a revision to our concepts of the mononuclear phagocyte system:

Questions: Where exactly do microglial progenitors fall into place in the new scheme of myeloid lineage commitment? Could microglia belong to a family of monocyte-derived dendritic cells, or do they derive from foetal macrophages originating from the yolk sac and liver?

Over the past few years, it has clearly emerged that circulating blood monocytes are themselves a heterogeneous population of cells, some of which give rise to cells which demonstrate features common to dendritic cells (Grage-Griebenow et al. 2001), as already summarised (refer to **Figure 105**). Whilst the evidence for duality in lineage commitment of CD34+ cord blood progenitors and circulating CD14+ monocytes appears convincing from tissue culture studies highlighted above, there is rather less certainty of the extrapolation of this system to events that occur during the development of tissue-resident mononuclear phagocytes. Based on their phenotypic and functional characteristics, it is almost certain that microglia derive from progenitors of the myeloid lineage. However, like dendritic cells, microglia downregulate their phenotype and are present within the CNS as 'immature' forms

which can be stimulated to 'mature' and adopt many functional and phenotypic characteristics normally attributed to macrophages. Therefore, the possibility that microglia are derived from immature bipotential progenitors with the ability to differentiate into, and adopt characteristics of macrophages and/or dendritic cells, remains to be explored (Flaris et al. 1993).

There have been several attempts to identify microglia-specific phenotypic markers, but these have been largely unsuccessful. With few exceptions (Graeber et al. 1989; Kida et al. 1993; Lassmann et al. 1993; Yokoo et al. 1998) antibodies raised against the mononuclear phagocyte population are generally present also on microglia, and identify these cells particularly when activated. From these studies, it would appear that parenchymal microglia exist in an 'immunologically immature' state, but do possess the potential to become fully differentiated phagocytic and antigen-presenting cells. This phenomenon, taken together with the restricted expression of CD45 and macrophage-related markers on microglia, has led some authors to the conclusion that microglia are derived from separate precursors to other populations of tissue-resident mononuclear phagocytes (Walker 1999). Several authors have noted that microglia possess functional and phenotypic similarities with professional antigen presenting dendritic cells (Fischer and Reichmann, 2001, Lowe et al. 1989, Penfold et al. 1993, Santambrogio et al. 2001, Ulvestad et al. 1994). Among these are their ability to process and present antigens, their expression patterns of 'empty' MHC class II molecules (Santambrogio et al. 2001), and B7 molecules (B7.2 is constitutively expressed by human microglia, and B7.1 can be expressed upon activation) (Becher and Antel, 1996), their profile of cysteine proteases (Santambrogio et al. 2001), their maturation upon exposure to T cells (Becher and Antel, 1996), expression of CD11c (Fischer and Reichmann, 2001), and reportedly, expression of dendritic markers CD1 (Lowe et al. 1989) and RFD1 (Ulvestad et al. 1994). Finally, osteopetrotic mice deficient in M-CSF, considered to be critical for driving the lineage of myeloid progenitors towards macrophages, still possess substantial numbers of mononuclear phagocytes within the brain (as well as in the lungs and liver) (Blevins and Fedoroff, 1995; Wegiel et al. 1998; Wiktor-Jedrzejczak et al. 1991; 1996; Witmer-Pack et al. 1993).

Likewise the possibility that parenchymal microglia represent a heterogeneous population of cells derived during development from foetal macrophages originating in the yolk sac and liver (and later from bone marrow-derived progenitors), warrants further investigation (Alliot et al. 1999; Shepard and Zon, 2000). Thus, foetal macrophage populations, derived from the yolk sac, liver and spleen, in the earlier stages of development which give rise to the resident microglial population, may differ in all likelihood from populations at later stages of development, and in the adult, that are mainly derived from bone marrow. This particular hypothesis is more compelling, given the nature of the colonisation of the developing human

and murine CNS by microglia during the period in development which corresponds to that when the liver is the principal haematopoietic organ (between 16-22GW in man, Torubarova et al. 1995). It would also explain differences between the initial wave of lectin positive (immunophenotype negative) progenitors that penetrate the CNS (particularly from the surrounding mesenchyme) and progressively differentiate into ramified microglia, and amoeboid cells which are derived concomitantly and at later periods from within blood vessels. These hypotheses remain to be established.

Concluding remarks

The present work has clearly defined the phasing and regional colonisation of the developing human and murine nervous systems by microglia. Colonisation of the developing human nervous system by microglia is an orchestrated response, related to the maturation of the nervous system, coincides with vascularisation of the CNS, is associated with radial glia, and correlates with the differentiation of astrocytes, neuronal migration and myelination, all of which are events that occur predominantly in the fourth and fifth months of life, and beyond. The sites of entry into the nervous system appear initially to derive from mesenchymal tissues (mesodermal progenitors/ foetal macrophages) that envelop the brain and spinal cord, and subsequently, at local vessels (within the corpus callosum and internal capsule for example, or in regions of the brain where apoptosis occurs), and from 'reservoirs' within the germinal layers, where large numbers of amoeboid microglia tend to accumulate. There are at least three successive stages in microglial colonisation of the human nervous system. First and foremost, progenitor cells migrate from the meninges/mesenchymal tissue surrounding the CNS and later, via blood vessels into the primary sites of colonisation: the intermediate zone and subplate, internal capsule, corpus callosum, ventricular and subventricular zones. Second, cells migrate tangentially along the germinal layers, within the corpus callosum, and radially along the direction of radial glial tracts spanning the intermediate zone (developing white matters) towards the cortical plate. Finally, microglia distribute ubiquitously throughout the CNS in non-overlapping fields. Colonisation of the spinal cord in man begins around 9GW, with the major influx of microglial progenitors commencing between 14-16GW. In the cerebrum, colonisation is well underway during the second trimester. Although the patterns of colonisation may appear to differ between the spinal cord and cerebrum, the route of traffic for these cells is the same: from white matter to developing grey areas. In the mouse, microglial progenitors begin to colonise the CNS around embryonic day 15, and these cells begin to differentiate to ramified forms from day 17 to day 19 onwards. The gradient of colonisation follows a caudal to rostral axis, in keeping with the general development of the nervous system, and the differentiation of these cells accompanies highly vascularised regions of the CNS.

The distribution of these cells is likely to be co-ordinated by regional and differential expression of chemokines such as MCP-1 (acting via CCR2), MIP-1α, RANTES, IL-8 and fractalkine in the cerebrum and by ICAM-2, PECAM and laminin on cerebral blood vessels and on capillaries within the germinal layer, as well as by regional apoptotic cell death (for example in the ventricular and subventricular zones). Developmentally regulated expression of ICAM-2 on vessels is significant, since this adhesion molecule is not inducible in inflammation and very low levels can be detected in the normal adult brain. A population of foetal microglia are actively dividing with the intermediate zone, and paired cells within this region express MIP-1α. Moreover, data from tissue culture experiments indicate that chemokines (such as MCP-1 and MIP-1α) may not only direct the migration of microglia and their progenitors, but may also influence the proliferation of developing astrocytes. The morphological status and motility of microglial progenitors are clearly influenced by the activity of astrocytes *in vitro*, and foetal microglia are closely associated with GFAP-positive radial glial cells, differentiating astrocytes and their processes *in situ* during the second trimester.

With new insights into the heterogeneity between foetal and adult macrophages, it is proposed that the initial phase of colonisation of the nervous system consists of foetal macrophages or their precursors that originate from the yolk sac and liver. This is followed by a secondary wave of colonisation by circulating progenitors derived from the foetal liver and subsequently bone marrow following the development of the vascular network and blood circulation. Therefore, amoeboid microglia may not represent the sole source for ramified microglia, and the precise nature of the earlier progenitors will require identification. At the end of this process, by the end of the second trimester (24GW) foetal microglia have established their territories, begin to downregulate many of their surface molecules and progressively lose their migratory capacity, amoeboid shape and transform to ramified microglia. Amoeboid microglia may be associated with modelling of the CNS architecture, similar to mononuclear phagocytes in other tissues (for example modelling of bone by osteoclasts during development).

Future directions

There is now considerable knowledge regarding the activation and involvement of microglia in the pathology of the CNS. By comparison, circumstances surrounding the origin, development, and normal resting state of these cells are less apparent. It is evident that microglial colonisation of the CNS and subsequent differentiation require specific temporo-spatial cues derived from their environment. Although astrocytes appear central to this process, there are indications that neurons also influence this behaviour. Chemokines as well as growth factors have emerged as important regulators driving the directed migration of microglia. However, the precise nature and roles of these and other factor during development will require confirmation. Likewise, the mechanisms governing the differentiation of microglia and their territorial phenomena await systematic investigation.

Future work should focus on the physico-anatomical relationship of microglial progenitors with regards to synaptogenesis and the development of the cerebrovasculature and leptomeninges (the meninges are indicated at 6GW, the choroid plexus appears around 7GW, and the dura mater and pia arachnoid are distinct from 8GW in man (Arey, 1965)). Most importantly, the concept of a dual origin for microglia (from mesenchymal progenitors/foetal macrophages, and subsequently from circulating blood progenitors or possibly derived from perivascular cells) will have to be addressed primarily in studies on developing rodents during the *foetal* as well as postnatal periods (for example in mice lacking the myeloid differentiation transcription factors PU.1 and CEBPα (Iwama et al. 1998, Tenen, 2001; Zhang et al. 1997), or in combined M-CSF/GM-CSF knockout animals (Lieschke et al. 1994, Seymour et al. 1997) which are deficient in or lack certain populations of mononuclear phagocytes, as well as through experimental manipulation of haematopoietic tissue sources). Furthermore, the focus for such investigations should be directed towards progenitors derived from the foetal liver, rather than the bone marrow, since this latter organ adopts the principal role of haematopoiesis from mid-trimester, when microglial progenitors are already disseminated throughout the nervous system. The use of knockout animals (e.g. osteopetrotic/M-CSF-/-, MCP-1/CCR2-/- and CD11b-/- mice: Jeetle et al. 2002) as well as transgenic mice (e.g. mice that overexpress MCP-1: Fuentes et al. 1995) will continue to present valuable tools to determine the signals driving the recruitment of microglial progenitors. The possible functions of developing microglia may be addressed further by examining non-inflammatory developmental disorders and malformation of the CNS, including those related to neuronal migration and enzymatic or lysosomal storage disorders.

The underlying purpose of this investigation was to understand the temporo-spatial phasing and mechanisms by which the nervous system is colonised by microglial progenitors. These data are of particular application to potential therapies for a number of paediatric disorders affecting the brain, including hereditary lipidoses (Niemann-Pick disease, Gaucher's disease, Krabbe disease and metachromatic leukodystrophy) (Scriver et al. 1989), which are caused by single enzyme deficiencies that may lead to the accumulation of lipids within mononuclear phagocytes and subsequently to progressive neuropathological damage of variable severity. These defects may be partly corrected by transplantation of normal bone marrow or cord blood (rich in haematopoietic progenitors). The use of microglial progenitors as a tool for gene therapy targeting the CNS in such disorders is now conceivable. This study has shown that an effective window for such therapy exists during the second trimester of intra-uterine human life, commencing with the infiltration of progenitors around the twelfth week of gestation.

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" I have endeavoured to show the reader the position in which our knowledge..... stood about the middle of the present year, and I trust that my book may enable many who have not yet attempted, but who are nevertheless anxious, to do something to advance this work a little nearer to its great therapeutic ends, to understand where to begin. If it be thought to fail in these objects, it may perhaps yet succeed indirectly, on the principle that a man who publicly upholds erroneous views often advances truth by the amount of opposition and discussion which he arouses. If even in this way, our knowledge ..... is in some small measure increased, the labour I have expanded upon this work may not have been altogether in vain."

W Ford Robertson, Edinburgh, 22nd October, 1900